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Enclosures

Development of a Highly Sensitive RT-PCR Luminometric Hybridization Assay for Human Telomerase Reverse-transcriptase Beta-plus Transcript

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Abstract. *Background:* Telomerase is a general diagnostic and prognostic molecular tumor marker since it is expressed in the majority of human tumors in contrast to most healthy tissues. *Alternate splicing of human telomerase reverse transcriptase (hTERT) has been shown to affect telomerase activity. Patients and Methods:* We have developed a hybridization assay that selectively detects the hTERT beta-plus transcript. Biotinylated PCR products were captured on streptavidin-coated microtiter wells, hybridized with digoxigenin-labeled probes and detected by a highly sensitive luminometric reaction. *Results:* The method was applied in ten colorectal tumor forceps biopsies and their corresponding normal tissues. Six out of ten tumors were positive for hTERT beta plus transcript whereas none of the corresponding normal tissues were found positive. There was a complete concordance between the hybridization assay and real-time PCR. When the method was applied in peripheral blood of 20 breast cancer patients with metastatic disease and 21 healthy blood donors, 14 patients (70%) were found positive while all 21 healthy blood donors were negative. *Conclusion:* The developed hybridization assay is highly sensitive and specific for the detection of hTERT beta-plus transcript in clinical samples.

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Key Words: hTERT beta-plus transcript, colorectal cancer, luminometric hybridization assay, real-time PCR.

Telomerase is a cellular ribonucleoprotein reverse transcriptase that synthesizes telomeric DNA onto chromosomal ends, using a segment of its own RNA as a template (1-3). A very strong association between telomerase activation and cell immortalization and malignancy has been established making this enzyme one of the most promising general tumor marker (4, 5) and target for cancer therapy (6). Moreover, telomerase activation was shown to additionally have a protective function that allows cell proliferation without requiring net lengthening of telomeres (7, 8). Two main components are required for core enzymatic activity of telomerase *in vitro*: human telomerase RNA (hTR), which contains the template for reverse transcription, and human telomerase reverse transcriptase (hTERT), which consists the enzyme's catalytic subunit (9). hTR is expressed in both telomerase negative and positive cells and is not a rate limiting unit of telomerase activity. On the contrary, expression of the functional hTERT protein is a prerequisite for acquisition of telomerase activity (10-12) and hTERT expression was identified as one of three fundamental genetic changes for human tumorigenesis, by the *in vitro* malignant transformation of normal human cells (13).

Interest in the potential application of telomerase as a diagnostic and prognostic tumor marker is growing steadily. This stems from the observation that greater than 85% of most human tumors express telomerase activity, whereas most healthy tissues do not (4, 5), the only exception being germ line cells and activated lymphocytes (14). The development of the sensitive "telomeric repeat amplification protocol" (TRAP) assay by Kim *et al.* (3, 15), in which the telomerase-synthesized DNA products are amplified by a subsequent polymerase chain reaction (PCR), enabled the wide screening of many clinical samples for the presence of telomerase activity. However, the quantitative determination of telomerase activity by the electrophoretic

and ELISA based TRAP assays has many limitations (16), since it requires enzymatically active specimens and RNA analysis conditions at the same time. This requirement sets serious limitations in the handling of many clinical samples on a routine basis and more importantly it is highly affected by the presence of protein activity inhibitors, proteases or RNAses in clinical samples. Recently our group has developed a quantitative luminometric hybridization assay for telomerase activity, based on the use of a specially designed DNA-IS (17).

The observation that functional hTERT protein is critical for acquisition of telomerase activity (9-12) has led to the wide application of hTERT mRNA determination through standard RT-PCR procedures for the identification of telomerase positive samples. Unlike telomerase activity, hTERT mRNA RT-PCR assay is insensitive to the presence of proteases and protein inhibitors. Essentially all major types of cancer have been screened for the presence of telomerase activity and hTERT mRNA in a variety of clinical specimens such as peripheral blood, tissues, fine needle aspirates, urine and bladder washings (4, 5). The development of quantitative RT-PCR assays for the determination of hTERT mRNA, based on real time PCR methodology, is a very important step towards automation and clinical applications of telomerase assays (18).

However, it is very important, especially for the diagnostic application of hTERT assays, to note that the presence of multiple alternatively spliced variants of hTERT, corresponding to the full length message as well as to spliced messages with critical reverse transcriptase motifs deleted has been recently shown (19-21). The hTERT transcript has been shown to contain at least six alternate splicing sites (four insertion and two deletion sites have been identified) (21). By using primers within the reverse transcription domain of hTERT, two splicing sites, α and β were found. Four splice variants, α^+/β^+ , α^-/β^+ , α^+/β^- , α^-/β^- , of 457 bp, 421 bp, 275 bp and 239 bp, respectively have been detected (20, 21). The α splice site causes a 36 bp (12 amino acid) deletion within the conserved reverse transcriptase motif A, and the hTERT alpha splice variant was found to be a dominant negative inhibitor of telomerase activity (22). The β splice site causes a 183 base deletion resulting in a nonsense mutation truncating the protein before the conserved reverse transcriptase motifs B, C, D and E, thus resulting in a non active TERT protein and catalytically inactive telomerase complex. This alternate splicing of hTERT transcripts prohibited the formation hTERT protein that contained functional reverse transcriptase domains and inhibited telomerase activity in non-cancerous cells (19). These posttranscriptional regulatory mechanisms must be seriously taken into account before designing a new assay for hTERT. Recently the hTERT transcript alternate splicing

variants were quantitated in different cell lines and compared to telomerase activity (21).

In the present study we report the development of a highly sensitive luminometric hybridization assay for the detection of hTERT beta plus transcript. We have used nested RT-PCR to achieve higher sensitivity and selective amplification of the hTERT mRNA region that includes the 183 bp region encoding for the B motif of reverse transcriptase (beta plus splice variant) (19-22) and real-time PCR to quantify this transcript. All hTERT alternate splice variants were also detected after amplification by nested RT-PCR. We have applied the developed methodology in forceps tumor biopsies and their corresponding adjacent and normal tissues of ten patients with colorectal carcinoma, as well as in peripheral blood mononuclear cells of twenty breast cancer patients with verified metastases and twenty one healthy blood donors.

Materials and Methods

Patients, tissues and cell culture. Forceps biopsy specimens were collected from ten colorectal carcinoma patients admitted to Saint Savas Hospital. For each patient, 3 specimens from colorectal tissue were obtained by microdissection, tumor tissue, tissue adjacent to the tumor and normal tissue. All samples were immediately stored in an RNA stabilizing solution (RNA later, AMBION, USA) and kept at -70°C until RNA extraction. Peripheral blood in EDTA was obtained from 20 patients with metastatic (stage IV) breast cancer admitted to the Medical Oncology Unit of "Helena Venizelou" hospital and 21 healthy volunteers. Peripheral blood samples from healthy donors and patients were collected and processed in the same manner. The peripheral blood mononuclear cells (PBMC) were isolated within one hour of venipuncture by gradient centrifugation with Ficoll Hypaque-1077 (Sigma Chemical Company, LTD, England), as previously described (23), and cell pellets were kept at -80°C until RNA extraction. All patients and donors gave their informed consent and the study has been approved by the Ethical and Scientific Committees of the participating Institutions. The human mammary carcinoma cell line MCF-7 was used as a positive control for the expression of hTERT mRNA. MCF-7 cells were grown in DMEM/Nut. Mix F12 (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 40 mg/L Gentamicin sulphate and quantitated by trypan blue exclusion using a hemocytometer as previously described (23).

Total RNA isolation and cDNA synthesis. Total RNA was isolated from samples using Trizol LS reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a chemical fume hood, under RNase free conditions. The isolated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -70°C until use. RNA concentration was determined by absorbance readings at 260 nm with the HITACHI UV-VIS (U-2000) spectrophotometer. RNA integrity and cDNA quality was tested for all samples by PCR amplification of the β -actin housekeeping gene as previously described (23) as well as by real

Table I. *Oligonucleotides used in this study.*

Name	Use	Oligonucleotide sequence ^a (5'-3')	Nucleotide position
LT5 ^b	Forward	CGGAAGAGTGTCTGGAGCAA	1784-1803
LT6	Reverse	GGATGAAGCGGATCTGGA	1910-1928
2164	Forward	GCCTGAGCTGTACTTTGTCAA	2164-2184
KAT4	Reverse	ACTTGCCCTGATGCGCA	2508-2525
2620	Reverse	CGCAAACAGCTTGTCTCCATGTC	2597-2620
LT-TERT	Hybridization probe ^c	TTCCCGATGCTGCTGACCTCT	1867-1888

a: hTERT mRNA, GeneBank Accession Number AF015950

b: Labeled with biotin

c: Labeled with digoxigenin

time PCR amplification of the human porphobilinogen deaminase (PBGD) mRNA as a housekeeping gene (LightCycler Telo TAGGhTERT quantificationkit, Roche Molecular Biochemicals). Reverse transcription was carried out with the THERMOSCRIPT RT-PCR System (Invitrogen, Life Technologies), according to the manufacturer's instructions. 1 µg of total RNA was used as starting material for cDNA synthesis.

Oligonucleotide sequences and labeling. All primers and hybridization probe sequences used in this study are listed in Table I. Oligonucleotide sequences were firstly evaluated in silico by using the PRIMER PREMIER 5 software (Premier Biosoft International, Palo Alto, CA, USA) in order to avoid primer-dimer formation, false priming sites and formation of hairpin structures. Oligonucleotides were synthesized at the Lab of Microchemistry of FORTH, (FORTH, Crete, Greece). Primer LT5, LT6, 2164 and 2620 sequences were taken from the literature (20). KAT4 primer was in-silico designed to anneal into the beta plus splicing region of hTERT mRNA while the hybridization probe LT-TERT was designed to anneal within the LT5/LT6 PCR product sequence. LT-TERT probe was tailed enzymatically with Dig-11-dUTP (Roche Molecular Biochemicals, USA), by using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals, USA), as previously described (17, 23) and used in the hybridization assay without further purification.

PCR amplification. All primers and hybridization probe positions as well as the PCR amplification and hybridization assay strategy are described in Figure 1. In this report, we refer to the hTERT cDNA that contains the 183 bp region (nucleotide positions 2342-2524) which is critical for reverse transcriptase activity of the hTERT protein (20), as hTERT beta plus transcript.

hTERT splicing variants. The distribution of hTERT mRNA alternate splicing variants in the samples was determined by nested RT-PCR. For the first PCR, primers LT5 and 2620 (20 pmol of each) and 1.5 µL cDNA were added in a total volume of 25 µL of PCR mixture. This first PCR yields a 837 bp product. An aliquot of 1.5 µL of the first PCR product was used as a template for the nested PCR using as inner primers the pairs 2164/2620 (20 pmol of each) giving four splicing products α^+/β^+ , α/β^+ , α^+/β^- , α/β^- , of 457, 421, 275 and 239 bp, respectively, detected by agarose gel electrophoresis (20). For both PCRs, the reaction mixture (25 µL)

consisted of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl₂ and 0.5 U Taq DNA polymerase (Platinum, Invitrogen, Life Technologies).

hTERT beta plus transcript. PCR amplification for hTERT beta plus transcript was also based on a nested PCR protocol. For the first PCR, primers LT5 and KAT4 (20 pmol of each) and 1.5 µL cDNA were added in a total volume of 25 µL of PCR mixture. This first PCR yields only the α^+/β^+ (742 bp) and α/β^+ (706 bp) hTERT products. An aliquot of 1.5 µL of the first PCR product was used as a template for the nested PCR using as inner primer pair the bLT5/LT6 (20 pmol of each) giving a single product of 145 bp. For both PCRs, the reaction mixture (25 µL) consisted of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2 mmol/L dNTPs, 2.5 mmol/L MgCl₂ and 0.5 U Taq DNA polymerase (Platinum, Invitrogen, Life Technologies). In both cases, the first PCR reaction was initiated by a 5 min denaturation at 94°C and terminated by a 5 min extension at 72°C. The cycling protocol (repeated 25 times) consisted of denaturation at 94°C for 30 sec, annealing at 60°C for 50 sec, and extension at 72°C for 50 sec. The nested PCR reaction was performed under the same conditions as above, the only difference being in cycling protocol (repeated 35 times). PCR amplification was performed in a Peltier Thermal Cycler (DNA Engine, PTC-200, MJ Research, USA). Amplified products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Hybridization assay. The principle of the developed luminometric hybridization assay for hTERT beta plus transcript is shown in Figure 1 and was similar to that previously reported (17, 23). Nested PCR products, biotinylated through the use of a biotin-labeled LT5 primer, were immobilized on streptavidin coated microtiter wells and detected through hybridization to a specially in-silico designed digoxigenin labeled probe, LT-TERT (Table I). Hybridized amplicons were detected using antidigoxigenin antibodies conjugated to alkaline phosphatase and Lumiphos, which is a highly sensitive chemiluminogenic substrate.

Opaque polystyrene microtiter wells (Microlyte 2, Dynatech, USA) were coated overnight at room temperature with 50 µL of streptavidin (1.4 mg/L) diluted in Phosphate-Buffered Saline (PBS, pH 7.4). During the whole assay the wells were washed three times after each step with wash solution [50 mmol/L Tris (pH 7.4), 0.15 mol/L NaCl and 1 mL/L Tween-20], using a microtiter plate washer

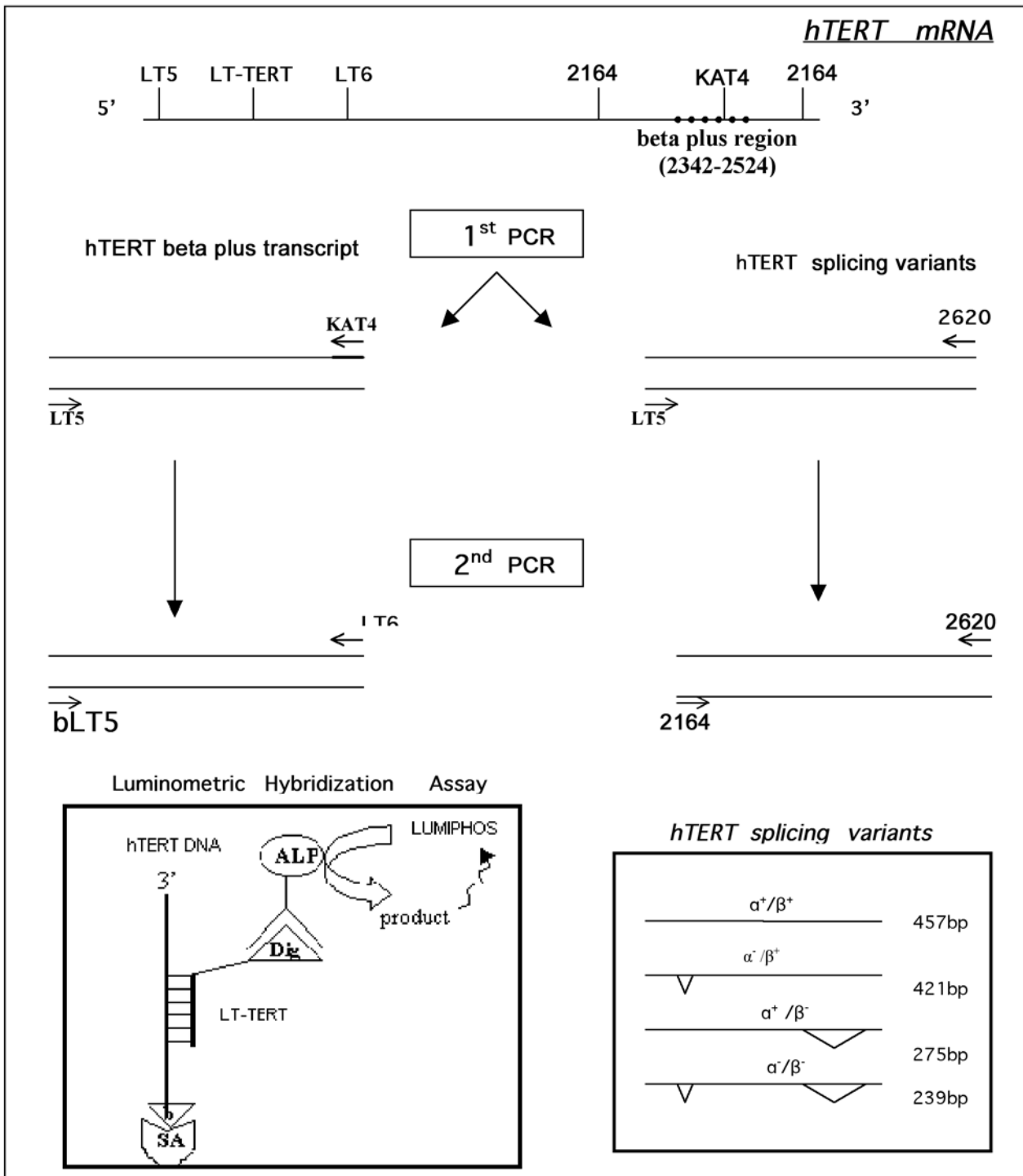


Figure 1. Detection scheme for hTERT alternate splicing variants and hTERT beta plus transcript by nested PCR and the proposed luminometric hybridization assay.

(Wellwash x4, Denley). Five μL of the biotinylated PCR products (bLT5/LT6 nested PCR, hTERT beta plus transcript) diluted 10-fold in blocking solution [10 g/L blocking reagent (Roche Molecular Biochemicals, USA) in 0.1 mol/L maleic acid and 0.15 mol/L NaCl, pH 7.5] were added per well, and incubated at room

temperature with shaking for 30 min. Subsequently, 50 μL of 0.2 mol/L NaOH were added. After a 20 min incubation at room temperature, the non-biotinylated DNA strands were removed by washing the wells. Fifty μL of a Digoxigenin-labeled probe (LT-TERT probe) (10 μM), 1000-fold diluted in blocking solution, were

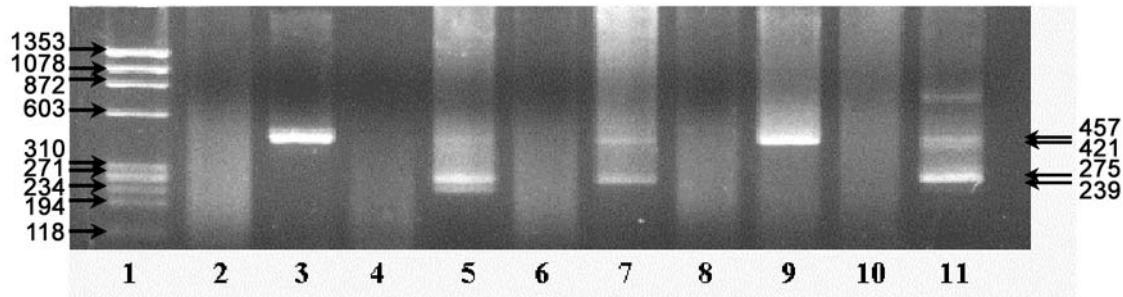


Figure 2. *hTERT* splicing variants in colorectal tumor biopsies. Lane 1: Φ X 174 DNA marker, lane 2: nested PCR blank, lane 3: #9, tumor, lane 4: #9 normal tissue, lane 5: #6, tumor, lane 6: #6, normal tissue, lane 7: #3 tumor, lane 8: #3 normal tissue, lane 9: #5 tumor, lane 10: #5 normal tissue, lane 11: MCF-7 cell line (positive control)

Table II. *hTERT* beta plus transcript expression and splicing pattern in colorectal carcinoma forceps biopsies ($n=3$).

Sample ID	Stage (TNM)	Hybridization assay (RLUs)	Real time RT-PCR ^a	$\alpha^+\beta^+$	$\alpha\beta^+$	$\alpha^+\beta^-$	$\alpha\beta^-$
1	T3N0M0	58,520	10.1	+	+	+	+
2	TN2M0	86,587	6.72	+	+	-	-
3	TN2M0	42,281	7.14	+	+	+	+
4	T4N0M0	150	n.d. ^b	-	-	-	-
5	TN1M0	80,431	13.3	+	+	-	-
6	T3N0M0	50,687	2.68	+	+	+	+
7	T3N0M0	255	n.d.	-	-	-	-
8	T3N0M0	223	n.d.	-	-	+	+
9	TN1M0	2,229	9.15	+	+	-	-
10	TN2M0	348	n.d.	-	-	-	-

a: (hTERT copies/PBGD copies)

b: not detected

added to each well and incubated at 55°C for 30 min. Fifty μ L of an anti-Digoxigenin antibody conjugated to alkaline phosphatase (750 U/L, Roche Molecular Biochemicals, 2000-fold diluted in blocking solution), were added per well and incubated at room temperature for 30 min. After this step, the wells were washed six times and 50 μ L of the chemiluminogenic ALP-substrate LumiPhosPlus (Aureon Biosystems, GmbH, Vienna, Austria) were added to each well. The enzymatic reaction was completed after a 30 min incubation at 37°C, in the dark. The chemiluminescence signal integral was measured in a Luminometer (Mediator PhL, MEDIATORS Diagnostika GmbH, Vienna, Austria) and reported as Relative Luminescence Units (RLUs).

Quantification of *hTERT* expression. Quantification of hTERT-encoding mRNA was performed in a real-time, one step RT-PCR using the LightCycler Technology and the LightCycler TeloTAGGG Quantification kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The calibration curve was constructed based on the use of RNA standards containing known copy numbers of hTERT mRNA, and their corresponding Cp values. For each sample, 1 μ g of total RNA was analyzed for both hTERT and porphobilinogen deaminase (PBGD) in separate RT-PCR reactions. A 198 bp fragment of the generated hTERT cDNA was amplified with specific primers. hTERT mRNA

expression in the unknown samples was calculated through a standard curve that was constructed from standards supplied with the kit. For quantification, hTERT values were corrected against the housekeeping gene PBGD. hTERT values are expressed as the ratio of hTERT mRNA to PBGD mRNA copy numbers respectively. Total RNA expressing hTERT was used as a positive control, while DEPC water was used as a negative control in each run.

Statistical analysis. The Wilcoxon test for paired non-normally distributed groups was used to test the significance of difference between the RLUs of colorectal tumors and their corresponding normal tissues. The Mann-Whitney test for unpaired non-normally distributed groups was used to compare the levels of hTERT beta plus transcript in the peripheral blood samples of breast cancer patients with those of the control population. Two-tailed tests were used at all times and statistical significance was set at $p < 0.05$. Data analysis was carried out with the Statmost statistical package (Statmost, DataMost Corp, USA).

Results

Primer selection and PCR optimization. For both nested PCR reactions, PCR conditions were optimized (MgCl₂ concentration, number of cycles, annealing temperature and amount of DNA sample). Hot start PCR was used in all cases for increased sensitivity and to avoid the formation of primer dimers. Especially for the selective amplification of hTERT beta plus transcript, in the presence of all other hTERT splice variants, primer KAT4 was in-silico designed to anneal onto the hTERT beta splicing region (2342-2524) and used as a reverse primer, in combination with LT5. The hTERT beta plus transcript amplified by this PCR is further amplified in a nested PCR by primers LT5 and LT6 (19). The combination of primers LT5 and 2620 (first PCR) and 2164-2620 (nested PCR) amplifies all hTERT splicing variants, since these primers anneal onto sequences that are common in all transcripts (Figure 1).

Luminometric hybridization assay. Hybridization assay conditions involving all steps such as reagent concentrations,

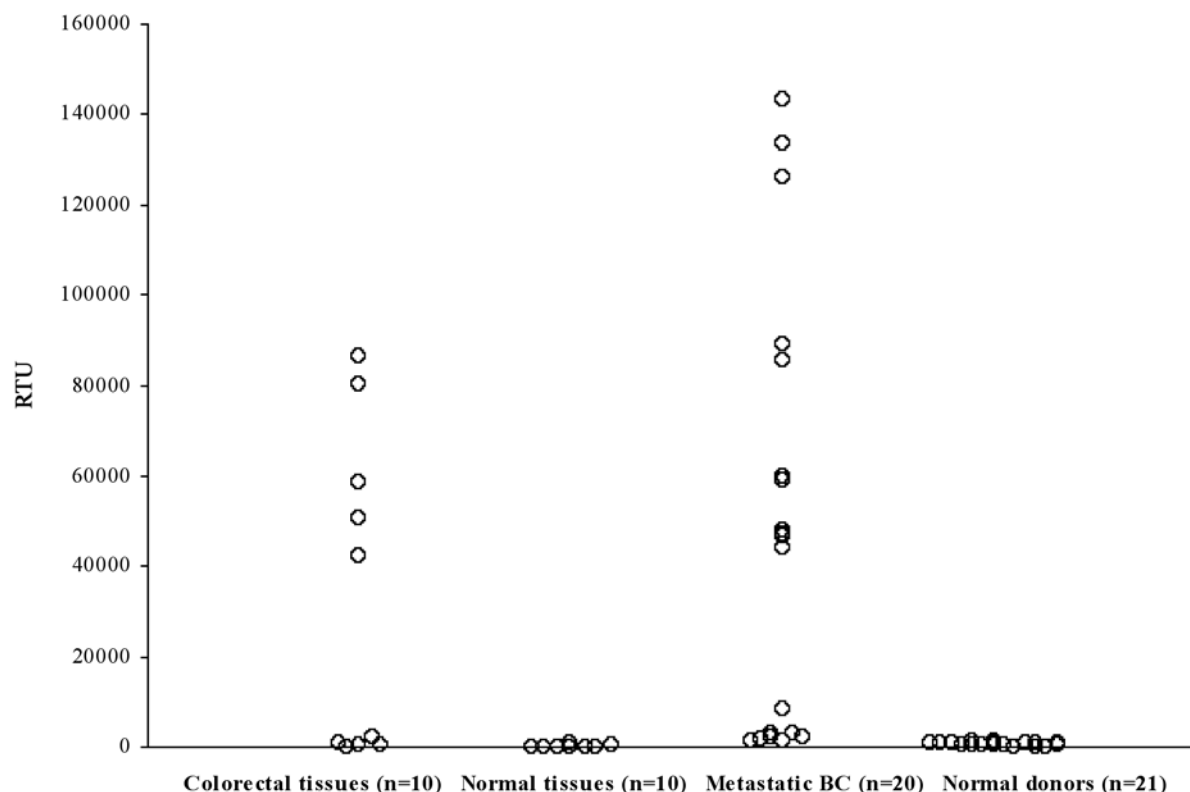


Figure 3. Expression of hTERT beta plus transcript in: a) Colorectal tissues (n=10), b) corresponding normal tissue, (n=10), c) PBMC of metastatic breast cancer patients (n=20), d) PBMC of healthy blood donors (n=21).

PCR product and labeled probe dilutions, annealing temperature and time of incubation periods were optimized in respect to the best signal to background ratios (S/B), sensitivity and rapidity (data not shown). The background (non-specific binding) is defined as the relative luminescence unit value (RLU) obtained when the nested PCR negative control was assayed. The detection limit was found to be 4 MCF-7 cell equivalents, since this concentration (RLUs: $34,612 \pm 2,485$, n=4) could be clearly distinguished from the background (RLUs: $1,098 \pm 168$, n=4), with a signal to background ratio of 31. The luminometric hybridization assay was more sensitive than agarose gel detection of nested PCR products, where 40 MCF-7 cell equivalents could be detected and this is in accordance with our previous results for CK-19 mRNA detection (23).

Within-run reproducibility of the hybridization assay was tested by analysing six times each of three different PCR products, corresponding to 4, 40 and 400 MCF-7 cell equivalents, and the corresponding CVs were found to be 8.4%, 6.5%, and 6.9% respectively. In order to evaluate the overall reproducibility (between-run) of the hTERT beta plus splice variant luminometric hybridization assay, five different MCF-7 cell suspensions isolated from the same number of MCF-7 cells (1×10^6 , as measured in a hemocytometer) were

subjected to the whole analytical procedure. This included all steps such as RNA extraction, cDNA synthesis, nested PCR and detection of biotinylated PCR products (in duplicate) by the proposed luminometric hybridization assay. In this case, the CV% was found to be 13.5 % (n=5x2).

hTERT splicing pattern in patients with colorectal carcinoma.

The proposed methodology was applied for the evaluation of hTERT splicing pattern in ten colorectal tumor forceps biopsies and their corresponding normal tissues. The same samples were analysed in triplicate for: a) hTERT beta plus transcript by the developed luminometric hybridization assay, b) quantification of hTERT-encoding mRNA by real-time, one step RT-PCR using the LightCycler TeloTAGGG Quantification kit and c) for the presence of all hTERT splice variants by nested RT-PCR and agarose-gel detection of the PCR products.

Clinical characteristics and hTERT splicing patterns for these samples as analyzed by this methodology are summarized in Table II. As can be seen in Table II six colorectal tumor tissue samples (#1, 2, 3, 5, 6, 9) were found positive for hTERT beta plus transcript (α^+/β^+ , α^-/β^+). However, the hTERT splicing pattern was different in these samples. More specifically samples 1 and 6 were positive for

all hTERT splice variants (α^+/β^+ , α^-/β^+ , α^-/β^- , α^+/β^-) while in samples # 2, 5 and 9 only the beta plus splicing variants (α^+/β^+ , α^-/β^+) were detected. Three samples (# 4, 7, 10) were found negative for all transcripts while sample 8 was positive only for the α^+/β^- and α^-/β^- splice variants, while negative by luminometric hybridization assay and real-time PCR. The characteristic hTERT splicing variants for some of these colorectal tumor biopsies can be seen in Figure 2. As can be seen in Table II, a complete concordance between the luminometric hybridization assay and quantitative real-time one step RT-PCR for hTERT mRNA was observed when the same samples were analyzed by the two methods.

The relative luminescence units (RLUs) corresponding to hTERT beta plus transcript in these ten colorectal tumor biopsies and their corresponding normal tissues differed significantly as can be seen in Figure 3. More specifically, the RLUs for the colorectal tumor samples [median (range): 22,255 (150-86,587) n=10] were significantly higher than those for the corresponding normal tissues [median (range): 216 (100-968), n=10]. Wilcoxon analysis test (two tailed) results showed a significant difference between tumors and normal samples ($p=0.007$).

Detection of hTERT beta plus splice variant in PBMC of patients with metastatic breast cancer. The proposed luminometric hybridization assay was applied for the detection of hTERT beta plus splice variant in PBMC of 20 patients with metastatic breast cancer and 21 healthy blood donors. The relative luminescence units (RLUs) corresponding to hTERT beta plus transcript between these two groups, as can be seen in Figure 3. More specifically, the RLUs for the metastatic breast cancer patients [median (range): 46,928 (1,401-143,310) n=20] were significantly higher than those for healthy blood donors [median (range): 600 (100-1,374), n=21], ($p<0.001$, Mann Whitney test). Among the 20 patients with metastatic disease 14 (70 %) were found positive for the presence of hTERT beta plus transcript in their peripheral blood mononuclear cells while all the 21 healthy blood donors tested were found negative.

Discussion

Interest in the potential application of telomerase as a diagnostic and prognostic tumor marker is growing steadily, since more than 85% of most human tumors express telomerase activity while most healthy tissues do not (4, 5). The conventional TRAP assay – widely used for the determination of telomerase activity – although very reliable and sensitive is quite laborious and time-consuming (3). Several modifications including novel primer design to improve the specificity of PCR amplification and hybridization assay formats with non isotopic detection schemes have improved the quantification of telomerase

enzymatic activity (15). Recently we reported the development of a highly sensitive luminometric hybridization assay for quantification of telomerase specific activity that was based on the use of a specifically designed DNA-IS (17). However, quantitative determination of telomerase activity has still many limitations since it requires enzymatically active specimens and RNA analysis conditions at the same time. This is very difficult when many clinical samples have to be analyzed on a routine basis. More importantly, determination of telomerase activity is highly affected by the presence of protein inhibitors, proteases or RNAses in clinical samples (16). The observation that the presence of catalytically active telomerase is closely related to hTERT expression (9-13) has led to many studies on the expression of hTERT mRNA in a variety of clinical specimens (14-18). The role of telomerase in colorectal cancer has been extensively studied during the last years (24-27). Moreover, the prognostic potential of hTERT in patients with colorectal carcinoma has been recently evaluated by real-time PCR analysis (28).

So far, most studies on the expression of hTERT in clinical specimens are based mainly on the amplification of hTERT by using primers that amplify all hTERT splice variants since they anneal onto a region common for all transcripts (14, 19, 28). However, according to recent studies, only the α^+/β^+ , α^-/β^+ splicing variants encode for catalytically active hTERT protein since they include the 183 bp sequence that is conserved and critical for all reverse transcriptases (20,21). The present methodology enables the detection of hTERT beta plus splice variant in clinical samples. The proposed hybridization assay is not quantitative, since it is based on luminometric detection of nested PCR products. By principle, nested PCR cannot be quantitative, since first PCR products have already reached the PCR plateau phase, before entering the second PCR. However, nested PCR was necessary to achieve the required sensitivity for hTERT beta plus splice variant in clinical samples. The luminometric hybridization assay described here, is highly specific by selectively amplifying the hTERT beta plus splice variant and highly sensitive as it can detect hTERT beta plus expression in total RNA derived from 4 MCF-7 cells. High sensitivity is achieved by the combination of nested PCR, enzymatic amplification and the use of a sensitive chemiluminescent reaction for monitoring the enzymatic activity. The proposed hybridization assay is performed in a simple way in microtiter wells with instrumentation commonly used for ELISA assays in many clinical laboratories. The method is more practical to perform than analysis based on gel electrophoresis since it allows simultaneous detection of large number of samples.

We have applied this methodology for the determination of the hTERT beta plus splice variants in forceps biopsies of 10 patients with colorectal cancer and their

corresponding normal tissues. Six out of ten tumor specimens were found positive for the presence of hTERT beta plus transcript while the corresponding normal tissues from the same patients were negative. It would be interesting to check for telomerase activity all these samples, and especially sample #8, which was found negative for the beta plus transcript, while positive for the beta minus transcripts. Unfortunately, this was impossible, because the amount of tissue sample available was really too small to perform two totally different sample extraction procedures, such as protein extraction for telomerase activity and total RNA extraction. A complete concordance between the proposed luminometric hybridization assay and quantitative real-time one step RT-PCR for hTERT mRNA was observed when these samples were analyzed by the two methods. In this way it can be used in case that real time PCR instrumentation is not available. When different primers that amplify all hTERT splice variants were used, seven tumor biopsies from the same patients were found positive. We have also applied this methodology for the determination of the hTERT beta plus splice variant in PBMC of 20 patients with metastatic breast cancer and 21 healthy blood donors. Among the 20 patients with metastatic disease 14 (70%) were found positive for the presence of hTERT beta plus splice variant in their peripheral blood mononuclear cells while it was not detected in none of the 21 healthy blood donors tested. This is in concordance with other studies (29-31) that evaluate telomerase mRNA as a molecular marker for metastasis. The presence of telomerase activity in peripheral blood of cancer patients can be an early indication of circulating tumor cells and micrometastasis, since peripheral mononuclear cells from healthy individuals have very low levels of telomerase activity in comparison to tumor cells. This appears to be a sensitive, specific and noninvasive approach for detecting circulating epithelial cancer cells in patients with metastatic breast cancer and could be of great value in monitoring the cancer cell proliferation during chemotherapy (29).

The RT-PCR luminometric hybridization assay described here provides a specific and sensitive method for the detection of hTERT beta plus transcripts in a variety of clinical specimens.

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