BRIEF METHODS

Real-Time Quantification in Plasma of Human Telomerase Reverse Transcriptase (hTERT) mRNA: A Simple Blood Test to Monitor Disease in Cancer Patients

Francisco Dasí, Salvador Lledó, Eduardo García-Granero, Roberto Ripoll, Maribel Marugán, Mar Tormo, Javier García-Conde, and Salvador F. Aliño

Departamento de Farmacología (FD, SFA), Facultad de Medicina, Universidad de Valencia; and Servicios de Cirugía (SL, EG-G, RR) y de Hematología y Oncología (MM, MT, JG-C) del Hospital Clínico Universitario de Valencia, Valencia, Spain

The recent report (Chen et al, 2000; Kopreski et al, 1999) that tumor-derived RNA is detectable in cell-free serum of cancer patients has opened up a new molecular approach for the early detection and follow-up of cancer. Because telomerase activity is the most general molecular marker for the identification of human cancer, the analysis of telomerase mRNA expression in serum is attractive as a potential tumor marker. Different genes encode for the various components of the human telomerase complex. Although a good correlation between telomerase reverse transcriptase (hTERT) expression and telomerase activity has been reported (de Kok et al, 1999), no quantitative studies in serum or plasma have been performed to date. The present study reports the development of a quantitative assay for the measurement of hTERT expression in the plasma of cancer patients based on real-time quantitative RT-PCR (qRT-PCR) normalized to the amount of RNA input using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Plasma hTERT mRNA was measured by qRT-PCR in 19 patients with colorectal cancer or follicular lymphoma, and in 10 healthy control subjects. The results show that this procedure highly discriminates between healthy subjects and cancer patients and strongly support the idea that a valuable diagnostic and prognostic test for cancer

might be developed using this genetic marker in plasma.

We analyzed tissues from excised primary colorectal cancer in 10 patients. The adjacent normal mucosa from the same patients was used as sources of normal RNA. Plasma and blood mononuclear cells were obtained from 18 cancer patients (9 with colorectal cancer and 9 with follicular lymphoma) and from 10 healthy subjects with no evidence of pathology, in 8-ml blood collection tubes containing sodium citrate gel and a density gradient medium (Vacutainer CPT #362761; BD, Franklin Lakes, New Jersey). RNA was isolated from 50 mg of tissue and 250 μ l of plasma in 1 ml of TRIZOL (Life Technologies, Paisley, United Kingdom) following the instructions of the manufacturer, with the addition of 5 μ g of RNase-free glycogen (GIBCO-BRL, Gaithersburg, Maryland) as carrier. RNA was further purified using the Nucleospin RNA II kit (Macherey-Nagel, Easton, Pennsylvania), which includes a DNase I treatment of the sample. DNA-free RNA was quantified spectrophotometrically. Purified RNA (200 ng) was reverse transcribed with the TaqMan Reverse Transcription reagents kit (Applied Biosystems, Foster City, California) according to the instructions of the manufacturer. After random hexamers annealed for 10 minutes at 20° C, cDNA synthesis was performed for 45 minutes at 42° C, followed by an inactivation step for 5 minutes at 95° C. For PCR analysis, 5 μ l of the RT reaction were mixed with 45 μ l of TaqMan Universal Master Mix containing 250 nm of the forward (hTERT-Fw: 5'-ACCGTCTGCGTGAG-GAGATC-3') and reverse (hTERT-Rv: 5'-CCGGTA-GAAAAAGAGCCTGTTC-3') primers and 125 nm of the TaqMan probe (hTERT-Probe: 5'-FAM-TGTACGT-CGTCGAGCTGCTCAGGTCTTT-TAMRA-3'). Primers

Received January 22, 2001.

This work has been supported by grants "Dr. Peset Aleixandre" and "Roig Giménez" and the IMTEFA 1999/52 project.

Address reprint requests to: Dr. Salvador F. Aliño, Department of Pharmacology, Faculty of Medicine, University of Valencia, Avda. Blasco Ibañez, 15, 46010 - Valencia (Spain). E-mail: alino@uv.es

and probe for hTERT were designed using the Primers Express software (Applied Biosystems). GAPDH primers and probes were from the TaqMan GAPDH control reagents kit (Applied Biosystems). PCR programming was 10 minutes at 95° C for enzyme activation, followed by 40 two-step cycles (15 seconds at 95° C; 1 minute at 60° C). All samples were measured in duplicate. The standard curve was prepared using the HT-29 colon carcinoma cell line. After cDNA synthesis, dilutions were made to produce a standard curve covering a concentration range of 0.016–250 ng. The cycle threshold (Ct) values of all samples were measured by the ABI Prism 7700 sequence detection system (Applied Biosystems) and were transformed to nanograms of HT-29 hTERT and HT-29 GAPDH using the standard curves measured in the same experiment. The ratio between ng of hTERT and ng GAPDH represents the normalized hTERT (hTERT_N) for each sample and can be compared with that of other samples (de Kok et al, 1999). The standard curves for each gene did not differ significantly between experiments, meaning that normalized hTERT values could be compared directly between experiments. TRAP assay (TRAP-eze Telomerase Detection Kit; Intergen, Purchase, New York) was performed according to the instructions of the manufacturer. Protein concentration was determined by the NanoOrange Protein Quantitation Kit (Molecular Probes, Eugene, Oregon).

To set up the qRT-PCR technique, hTERT_N expression was measured in tumor samples and adjacent normal mucosa from 10 colorectal cancer patients and results are summarized in Table 1. hTERT_N was significantly higher ($p < 0.02$) in tumor tissues (20.81 ± 5.36) than in the corresponding normal tissues (5.50 ± 1.27). The quantitative data showed perfect correlation with the semiquantitative TRAP assay (Fig. 1), but the measurement of hTERT expression by qRT-PCR has several marked advantages over the TRAP assay for cancer detection: (a) the assay output is numerical rather than qualitative, allowing appropriate diagnostic statistics to be applied; (b) it has higher sensitivity and improved resistance to plasma inhibitors; (c) it uses endogenous controls which allow correction for parameters like RNA input, RNA degradation, or RT inhibitors; and (d) the closed-tube method minimizes the risk for cross-contamination.

hTERT_N expression was also measured in plasma and blood cells of 10 healthy volunteers and compared with that of 9 colorectal cancer patients and 9 follicular lymphoma patients. The mean values of plasma hTERT_N expression in colorectal cancer (18.97 ± 4.91) and follicular lymphoma (26.27 ± 5.81) patients were significantly higher ($p < 0.005$) than in healthy donors (2.71 ± 0.41), but no significant correlation was observed between tumor stage (pTNM) and the amount of plasma hTERT (data not shown). In addition, when comparing individual data (healthy subjects vs cancer patients), we observed that 8 of 9 colorectal cancer patients and 9 of 9 follicular lymphoma patients showed hTERT_N values higher than the maximum value (4.82) observed in the control group. hTERT_N expression in blood cells of colorectal

Table 1. Expression of Normalized hTERT (hTERT_N) in Plasma, BMC, and Colorectal Tissues^a

Sample number	Plasma				BMC				Colon tissue ^b			
	Healthy hTERT _N	CRC hTERT _N	pTNM	Lymphoma hTERT _N	Ann Arbor Stage	Healthy hTERT _N	CRC hTERT _N	pTNM	Lymphoma hTERT _N	Ann Arbor Stage	Healthy hTERT _N	Tumor hTERT _N
1	1.08	0.93	T3N0M0	10.52	III	0.68	0.05	T3N0M0	9.55	III	1.36	15.72
2	1.08	7.19	T1N0M0	11.03	III	1.40	0.52	T3N1M1	71.29	III	2.00	9.00
3	1.40	10.76	T3N0M0	14.59	IV	3.20	2.50	T3N0M0	79.36	III	2.51	19.20
4	2.44	11.52	T4N0M0	16.23	III	7.00	3.00	T3N0M0	131.10	III	3.67	4.74
5	2.46	12.56	T3N0M0	18.67	II	8.24	5.00	T3N0M0	459.21	IV	3.75	5.90
6	2.46	15.89	T3N0M0	28.13	III	15.00	7.13	T1N0M0			4.01	31.00
7	3.54	32.87	T3N1M1	28.38	IV	17.43	10.26	T4N0M0			4.27	5.41
8	3.75	33.98	T3N0M0	48.87	IV	24.17	70.40	T1N0M0			10.41	32.32
9	4.09	45.11	T1N0M0	60.01	IV	33.93	271.46	T3N0M0			10.50	58.71
10	4.82					108.71					12.56	26.13
Mean ± SE	2.71 ± 0.41	18.97 ± 4.91*		26.27 ± 5.81*		21.97 ± 10.21	41.14 ± 29.72**		150.10 ± 79.63**		5.50 ± 1.27	20.81 ± 5.36***

BMC, blood mononuclear cells; CRC, colorectal cancer; hTERT, human telomerase reverse transcriptase.

^aNormalized hTERT = $100 \times (\text{hTERT/GAPDH})$. Pathological data of CRC (pTNM) and lymphoma (Ann Arbor Stage) are included.

^bEach line represents the hTERT_N values derived from the healthy and tumor tissue from the same patient.

* $p < 0.005$ compared with healthy plasma group; ** $p > 0.05$ compared with healthy BMC; *** $p < 0.02$ compared with healthy tissue. Statistical significance evaluated by Student *t*-test.

Figure 1.

Detection of telomerase activity in colorectal cancer patients. Representative TRAP gel of normal mucosa (N) and their corresponding tumor (T) tissues from four colorectal cancer patients is shown. +, positive control of 500 HT-29 cells; -, negative control consisting of 500 HT 29 cells, heated at 96° C for 10 minutes to inactivate telomerase activity. IPC, internal positive control.

cancer (41.14 ± 29.72) and follicular lymphoma patients (150.10 ± 79.64) was higher than in healthy donors (21.97 ± 10.21), but statistically significant differences were not observed. Thus, the analysis of individual data from blood cells lacks interest because of the high deviation observed, and this reinforces the interest in plasma analysis. To our knowledge, this is the first report that telomerase mRNA can be quantified in the plasma of cancer patients and opens the way for a new noninvasive blood test for cancer.

Most cancer cells, exhibiting immortal growth, present increased telomerase expression compared with previously normal tissue or quiescent cells. Recent evidence indicates that human cell growth is ultimately limited by telomerase reverse transcriptase and that telomerase expression can endow cells with unlimited proliferative potential.

Human cells can also use a non-telomerase-based mechanism, alternative lengthening of telomers (ALT), probably involving recombination, to maintain telomers. However, the ability of human cells to activate this ALT mechanism is very poor (Chin et al, 1999), and the results showing that human cancer cells in culture die after the inhibition of telomerase (Herbert et al, 1999) support the idea of a central role for telomerase in endowing cells with immortal growth. Telomerase shortening in primary cells leads to senescence (Bodnar et al, 1998), though continued telomere attrition and loss of telomere capping activity (mainly because of the neutralization of Rb and p53 functions) result in severe chromosomal instability and the massive cell death referred to as "crisis" (Counter et al, 1992). In cancer patients, we could expect a significant proportion of the nucleic acid in plasma to be derived from tumor cell release or destruction. Consequently, real-time hTERT quantitation in plasma could be viewed as a promising tumor marker. Our preliminary results show that real-time measurement of hTERT mRNA in plasma discriminates between healthy subjects and cancer patients suffering from colorectal cancer or follicular lymphoma and support the idea that a diagnostic or prognostic test for cancer might be developed based on this genetic marker in blood. This observation agrees with previous studies suggesting that RNA might be present in plasma or serum (Wieczorek et al, 1985, 1987) and with more

recent reports that human mRNA of tyrosinase (Kopreski et al, 1999) and telomerase (Chen et al, 2000) can be extracted and amplified from the serum of patients with malignant melanoma and breast cancer, respectively. Here, we have shown that real-time PCR of hTERT could be used in the future and might increase the number of cases detected by revealing the number of copies in healthy persons and in cancer patients, particularly when considering that the presence in plasma of tumor-derived mRNA is not limited to any given cancer type but may be a relatively frequent finding in a wide range of cancers. The procedure opens a new avenue in quantitative plasma analysis because it could be extended to measure other mRNAs in plasma, although further studies are necessary to validate the specificity and sensitivity of the assay.

References

- Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, and Wright WE (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* 279:349–352.
- Chen XQ, Bonnefoi H, Pelte M-F, Lyautey J, Lederrey C, Movarekhi S, Schaeffer P, Mulcahy HE, Meyer P, Stroun M, and Anker P (2000). Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res* 6:3823–3826.
- Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW, and DePinho RA (1999). p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* 97: 527–538.
- Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, and Bacchetti S (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 11:1921–1929.
- de Kok JB, Zendman AJ, van de Locht LT, Ruers TJ, van Muijen GN, Mensink EJ, and Swinkels DW (1999). Real-time hTERT quantification: A promising telomerase-associated tumor marker. *Lab Invest* 79:911–912.
- Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, and Corey DR (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci USA* 96:14276–14281.
- Kopreski MS, Benko FA, Kwak LW, and Gocke CD (1999). Detection of tumor messenger RNA in the serum of patients with malignant melanoma. *Clin Cancer Res* 5:1961–1965.
- Wieczorek AJ, Rhyner C, and Block LH (1985). Isolation and characterization of an RNA-proteolytic complex associated with the malignant state in humans. *Proc Natl Acad Sci USA* 82:3455–3459.
- Wieczorek AJ, Sitaramam V, Machleidt W, Rhyner K, Perruchoud AP, and Block LH (1987). Diagnostic and prognostic value of RNA-proteolipid in sera of patients with malignant disorders following therapy: First clinical evaluation of a novel tumor marker. *Cancer Res* 47:6407–6412.