

*Advances in Brief***Telomerase RNA as a Detection Marker in the Serum of Breast Cancer Patients¹**

Xu qi Chen, Hervé Bonnefoi,
Marie-Françoise Pelte, Jacqueline Lyautey,
Christine Lederrey, Sina Movarekhi,
Pierre Schaeffer, Hugh E. Mulcahy, Pierre Meyer,
Maurice Stroun, and Philippe Anker²

Faculté des Sciences, Pavillon des Isotopes, 1211 Geneva [X. q. C., J. L., C. L., M. S., P. A.]; Département de Gynécologie, Hôpital Cantonal Universitaire, 1211 Geneva [H. B., S. M., P. S.]; Département de Pathologie, Faculté de Médecine, Université, 1211 Geneva [M-F. P.]; Département de Chirurgie, Hôpital de la Tour, 1217 Geneva [P. M.], Switzerland; and Digestive Diseases Research Centre, St. Bartholomew's Hospital, London E1 2AD, United Kingdom [H. E. M.]

Abstract

Tumor-derived circulating DNA has been found in the plasma of cancer patients. Alterations include decreased strand stability, mutations of oncogenes or of tumor suppressor genes, microsatellite alterations, and hypermethylation of several genes. RNA has also been found circulating in the plasma of normal subjects and cancer patients. Tyrosinase mRNA has been extracted from the serum of melanoma patients and subjected to RT-PCR. Moreover, the presence of cell-free EBV-associated RNA has been reported in the plasma of patients with nasopharyngeal carcinoma. Human telomerase comprises two RNA subunits, telomerase RNA template (hTR) and its catalytic component, telomerase reverse transcriptase protein (hTERT). Expression of these subunits correlates with telomerase activity. Using RT-PCR, we investigated whether these RNA subunits were present in the serum of 18 patients with breast cancer, 2 patients with benign breast disease, and 21 normal subjects. The presence of amplifiable RNA was confirmed in all tissue and serum samples using RT-PCR of glyceraldehyde-3-phosphate dehydrogenase RNA. hTR was found in 17 of 18 tumors (94%) and 5 of 18 serum samples (28%). hTERT was also detected in 17 of 18 tumors (94%) and in 4 of 16 available serum samples (25%). hTR and hTERT were undetectable in tissues and sera taken from 2 patients with benign disease and in the sera of 21 normal subjects. We conclude that RNA is detectable in the serum of breast cancer patients and that

tumor-derived mRNA can be extracted and amplified using RT-PCR, even in patients with localized disease. This may have implications for cancer diagnosis and follow-up in the future.

Introduction

Adenocarcinoma of the breast is the most common malignancy in the female Western population and is responsible for approximately one in five cancer-related deaths among American women (1). Long-term survival following breast cancer improved little during the 1970s and 1980s (1), although diagnostic and therapeutic advances have since resulted in mortality reductions over the past 10 years (2). A potentially important strategy to reduce breast cancer mortality has been the introduction of mammographic screening to detect asymptomatic disease. Several studies indicate that mass screening can reduce breast cancer mortality (2–7). However, there are disadvantages associated with this form of screening, including a high rate of false positive tests, frequent false negative results, and considerable implications for public health spending (8–11).

Gene alterations identical to those found within the primary tumor have been detected in extracellular circulating DNA extracted from the plasma and serum of breast cancer patients. These include microsatellite instability or loss of heterozygosity (12–15), *p53* gene alterations (16), and aberrant methylation of the *p16^{INK4a}* gene (12, 17). Such data, combined with the results obtained from the plasma and sera of patients with other cancers (18), suggest that a diagnostic or prognostic test for cancer might be developed using genetic markers within blood. In addition to DNA, RNA has also been found circulating in the plasma and sera of normal subjects and in those with cancer. Kamm and Smith (19) found high concentrations of RNA in the plasma of healthy individuals, and Wiczorek *et al.* (20–22) reported similar findings in a variety of cancer types. More recently, tyrosinase mRNA has been detected convincingly in the plasma of malignant melanoma patients (23), and cell-free EBV-associated RNA has been detected in the plasma of patients with nasopharyngeal carcinoma (24).

Eukaryotic chromosomal ends consist of repeating DNA sequences (TTAGG) termed “telomeres.” These stabilizing terminal sequences become progressively shortened during each cell cycle, eventually resulting in cellular death. Telomerase is a ribonucleoprotein enzyme that adds telomeric repeats onto chromosomal ends, thereby replacing the lost DNA. Telomerase is composed of two core enzymatic subunits, hTR³ and hTERT. hTR and hTERT expression is related to telomerase activity (25–29). Thus, normal somatic cells have low or undetectable

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² To whom requests for reprints should be addressed, at Pavillon des Isotopes, 20 bvd d'Yvoy, Genève 1211, Switzerland. Phone: 42-22-702-63-37; Fax: 42-22-781-51-93; E-mail: anker@sc2a.unige.ch.

³ The abbreviations used are: hTR, telomerase RNA template; hTERT, telomerase reverse transcriptase protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

telomerase levels whereas cancers, including breast cancer, have detectable telomerase activity in 85–100% of cases (30). In this study, we report the presence hTR and the catalytic subunit hTERT in both tumor and serum samples taken from breast cancer patients.

Materials and Methods

Patients. Eighteen informed and consenting female patients (ages 47–87 years) undergoing surgery for breast cancer at the Hôpital Cantonal Universitaire, Geneva, between October 1999 and June 2000 were included in this study. Tumor samples were collected at the time of surgery and histopathologically characterized to confirm the diagnosis. Clinical and pathological data, including patient age, tumor size, lymph node status, histological type, and histoprognostic grade were also collected. Two patients undergoing surgery for benign breast disease (ages 47 and 76 years) were included in this study. Twenty-one normal subjects including seven males (ages 30–73 years) were studied as negative controls.

Sample Collection. Blood was collected prior to surgery in plain tubes for serum sampling. After clotting, tubes were centrifuged at $900 \times g$ for 15 min at room temperature, and the serum was collected. This was followed by a second 15-min centrifugation at $900 \times g$ to remove cellular debris. Serum samples were aliquoted and stored at -70°C until use. Tumor samples were removed at the time of surgery. The tumor was histopathologically characterized to confirm its diagnosis. A small portion was taken for study purposes, part being used for RNA extraction and part being stored at -70°C for additional extractions.

RNA Extraction. RNA was extracted using a commercially available kit (SV Total RNA Isolation System; Promega, Madison, WI), according to the manufacturer's instructions with a slight modification for serum samples. To each 100 μl of serum was directly added 175 μl of SV RNA lysis buffer. Only fresh or once-frozen thawed serum was used.

RT-PCR for hTR, hTERT, and GAPDH RNA. The Qiagen One Step RT-PCR kit (Qiagen, Basel, Switzerland) was used to detect the presence of hTR, hTERT, and GAPDH RNA in tumor and serum. For hTR analysis, 1 ng of tumor RNA was used in a 25- μl RT-PCR reaction mixture containing 400 μM each of deoxynucleotide triphosphate, Omniscript reverse transcriptase (designed for all reverse transcription with any amount of RNA from 50 ng to 2 mg/reaction), Sensiscript reverse transcriptase (optimized for very small amounts of RNA, <50 ng), hot-start Taq DNA polymerase, and 0.15 μM primers P1 and P2. The mixture of the two systems was found empirically to increase sensitivity. In the case of serum RNA, the quantity being too small to be measured by UV, two concentrations were used each time corresponding to 1 and 5 μl of the extract because sometimes 1 μl was not sufficient. The RT-PCR conditions were an initial incubation at 50°C for 30 min followed by a 95°C incubation for 15 min to activate the HotstarTaq DNA Polymerase, then 50 cycles at 94°C (30 s), 65°C (1 min), 72°C (1 min), and a 10-min final extension at 72°C . RT-PCR conditions were identical for the detection of hTERT, except that 0.3 μM primers P3 and P4 were used. GAPDH was used as a PCR quality control. Again, control PCR conditions were identical,

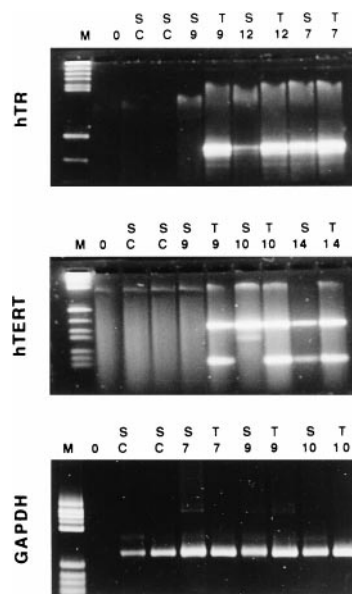


Fig. 1. Gels of hTR, hTERT, and GAPDH RT-PCR products. M, molecular weight marker; S, serum; T, tumor; C, healthy control; 7, 9, 10, 12, 14, patient numbers.

except that 0.075 μM primers P5 and P6 were used in the RT-PCR reaction.

Primers used were as follows: (a) for hTR (sense), GAAGGGCG-TAGGCGCCGTGCTTTTGC and (antisense), GTTTGCTCTAGAATGAACGGTGAAGG; (b) for hTERT (sense), TGACACCTCACCTCACCCAC and (antisense) CAC-TGTCTCCGCAAGTTCAC; and (c) for GAPDH (sense), CG-GAGTCAACGGATTTGGTCGTAT and (antisense) AGCCT-TCTCCATGGTGGTGAAGAC.

PCR amplification yielded products of 111 bp for hTR, 95 bp for hTERT, and 308 bp for GAPDH RNA. Products were run at 55°C on Elchrom Scientific S-50 gels (Elchrom Scientific, Cham, Switzerland), stained with SYBR-gold (Molecular Probes, Eugene, OR) for 45 min, and destained twice in a darkroom for 30 min with deionized water. Molecular analyses were performed by personnel blinded to clinical and pathological data, and all experiments were repeated at least once. A number of samples appeared positive initially, but were negative thereafter. Only samples clearly positive twice were scored as positive. Fisher's exact test and Wilcoxon's rank-sum test were used for the statistical analysis of categorical and continuous data, respectively.

Results

Of the 20 patients undergoing surgery for suspected breast cancer, 2 had small benign tumors which were unavailable for study. The presence of tissue mRNA was therefore assayed in 18 tumors, 2 cases of benign disease, and 20 serum samples. GAPDH RNA was detected in the tumors and serum samples of all cancer patients, in the serum of all patients with benign disease, and in negative controls (Fig. 1).

hTR was detected in 17 of 18 tumors studied (94%) and in 5 serum samples (28%; Table 1 and Fig. 1). The single-case

Table 1 hTR and hTERT in serum and tumor with clinical data concerning 18 breast cancer patients and 2 patients with benign breast disease

Patient no.	Age	Type ^a	pTNM	hTR		hTERT	
				T ^b	S	T	S
1	68	IDC	pT ₄ G ₂ N ₀	+	-	+	NA
2	48	IDC	pT _{1c} G ₂ N ₀	-	-	+	+
3	62	IDC	pT _{1c} G ₁ N ₀	+	-	+	-
4	54	IDC	pT _{1b} G ₂ N ₀	+	+	+	NA
5	76	IDC	pT ₂ G ₃ N _{1biii}	+	+	+	-
6	71	ILC	pT ₂ G ₂ N ₀	+	+	+	-
7	69	IDC	pT _{1c} G ₁ N ₀	+	+	+	+
8	59	ILC	pT ₂ G ₁ N ₀	+	-	+	-
9	73	IDC	pT _{1c} G ₁ N ₀	+	-	+	-
10	56	IDC	pT ₂ G ₂ N _{1biii}	+	-	+	+
11	61	ILC	pT ₂ G ₂ N _{1biii}	+	-	-	-
12	67	IDC	pT _{1b} G ₃ N ₀	+	+	+	-
13	86	IDC	pT ₂ G ₃ N ₀	+	-	+	-
14	87	IDC	pT _{4b} G ₂ N ₀	+	-	+	+
15	67	IDC	pT _{1b} G ₁ N ₀	+	-	+	-
16	57	IDC	pT _{1c} G ₂ N ₀	+	-	+	-
17	76	BD		NA	-	NA	-
18	78	PCIS	pT _{1c} G ₃ N _x	+	-	+	-
19	47	IDC	pT ₂ G ₃ N _{bi}	+	-	+	-
20	47	BD		NA	-	NA	-

^a IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PCIS, papilloma carcinoma *in situ*; BD, benign disease.

^b T, tumor; S, serum; NA, not available.

negative for tumor hTR was also negative for serum hTR. In addition, hTR was not found in serum samples from 21 healthy subjects or in the 2 patients with benign breast disease. As with hTR, the sera of all 21 healthy subjects and the two patients with benign disease were negative for hTERT (Table 1 and Fig. 1). In contrast, hTERT was detected in 17 of 18 tumors (94%). The single-case negative for tumor hTERT was also negative for serum hTERT. Two serum samples were unavailable for hTERT analysis. Of the remaining 16 serum samples, 4 (25%) were hTERT positive [Fig. 1, see *upper band* at 95 bp, the *lower band* (<50 bp) being probably a primer dimer].

In three cases, serum samples that were hTR negative were hTERT positive. Therefore, at least one telomerase RNA subunit was present in the sera of 8 of 18 breast cancer patients (44%). The presence of telomerase subunits was unrelated to patient age ($P = 0.97$), tumor size ($P = 0.68$), tumor grade ($P = 0.54$), or the presence of nodal metastases ($P = 0.67$).

Discussion

Kopreski *et al.* (23) have recently detected human tyrosinase mRNA sequences in the sera of four of six patients with metastatic malignant melanoma. This tumor-derived mRNA was extractable from plasma even after the blood was filtered to remove intact malignant cells, indicating that the mRNA was extracellular. Moreover, Lo *et al.* (24) have found the presence of cell-free EBV-associated RNA in the plasma of patients with nasopharyngeal carcinoma. Our study shows that tumor mRNA is not only amplifiable from the sera of patients with advanced disease, but that this RNA is also present in the serum of breast cancer patients bearing small, well differentiated tumors (T₁G₁) without either nodal or distant spread. We used an

RT-PCR assay to detect telomerase subunits and, as with Kopreski's study, we did not detect these in the sera of normal subjects or in patients with benign breast disease. In contrast, at least one subunit was present in 44% of sera from cancer patients.

This is the first time that tumor-related RNA has been reported in the sera of breast cancer patients. Tumor DNA has already been reported in the plasma or the sera of breast cancer patients (12–17) using microsatellite, p53 mutations or p16 hypermethylation. No single marker has yielded such a high percentage of positive cases. To reach 66% of the plasmas of breast cancer patients bearing a tumor-related DNA alteration, Silva *et al.* (12) had to use 6 microsatellite markers, p53 mutations, and methylation patterns of the first exon of p16.

The presence of hTR and hTERT in the serum is probably the reflexion of the expression of this RNA which took place in the tumor cells. Expression of hTR and hTERT has been reported to be observed specifically in telomerase-positive cancer cell lines and clinical cancer tissues (25–29). The hTERT and hTR subunits are independently expressed *in vivo*, and little is known about the mechanism of their assembly (27). This might explain the discordance of hTERT and hTR results in the sera of several patients. This kind of discordance has already been observed by others (25, 27, 28).

Telomerase activity is not observed easily in the serum due to the degradation of the RNA components of the nucleoprotein. However these RNA components are still large enough to be put in evidence with a RT-PCR, resulting in small PCR products. The PCR conditions we used (50 cycles) were adapted for the analysis of serum DNA. It is interesting to note that in the same conditions, all lymphocytes, whether they came from healthy controls or from patients, were positive (results not shown). This is not surprising, because telomerase activity has been reported in normal lymphocytes (31). But with this simple method, all sera from healthy controls were negative, and an important percentage of cancer patient sera were clearly positive. Of course, real time PCR could be used in the future and might increase the amount of cases detected by giving the number of copies in healthy persons and cancer patients.

Telomerase RNA might serve as a useful cancer marker in the future. Previous studies have shown that telomerase activity is an extremely frequent finding in neoplastic disease, and serum analysis might therefore be applied to the detection of a broad range of cancers. Indeed, we have detected hTR and hTERT in the sera of five of five patients with gastrointestinal malignancies (data not shown). This indicates that the phenomenon of circulating extracellular tumor-derived mRNA is not confined to any one cancer type, but may actually be a relatively ubiquitous finding across a broad range of cancers at various pathological stages.

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