Colorectal carcinoma (CRC) ranks second in death due to cancer in Western countries. Although surgical resection and adjuvant therapy are effective curative treatments, the risk of disease recurrence cannot be foreseen, even in patients at the same tumor stage. Therefore, it is important to identify biological prognostic variables, particularly for early-stage tumors in which the therapeutic approach might be improved.

Colorectal carcinogenesis is a multistep process, from adenoma to invasive carcinoma, for which several genetic events have been characterized; consequently, genetic alterations involved in the development of CRC have been proposed as potential markers of disease progression. Among them, microsatellite instability, K-ras, and p53 gene mutations have been extensively studied to evaluate their prognostic and predictive role in gastrointestinal cancer, but a definitive agreement has not yet been reached (1–3).

Great efforts have also been made to identify noninvasive or minimally invasive tumor markers for the detection of CRC in its early stages. Genomic mutations have been reported to be useful for the detection of either circulating tumor cells or tumor-derived DNA in serum and plasma (4). Evaluation of tissue-specific gene expression has been proposed as an alternative approach for identifying circulating tumor cells (5). Expression of cytokeratins as a marker of epithelial origin of tumor cells and expression of carcino-embryonic antigen have been used primarily to detect CRC cells in the haemopoietic milieu. Nonetheless, the diagnostic/prognostic role of all these markers is still to be established (6). More recently, the detection of cancer-related RNA molecules in plasma has been proposed as a tool for cancer diagnostics. Although stability and integrity of free RNA may be impaired by RNases, circulating RNA is partially protected, most likely by inclusion within apoptotic bodies (7, 8). RNA can be isolated from plasma, and plasma-derived RNA can be reverse transcribed and amplified by PCR (7, 9). Recently, analysis of circulating RNA has been also reported as a potential approach for gene expression profiling in cancer patients (10).

Telomerase, a ribonucleoprotein complex containing an internal RNA template (hTR) and a catalytic protein with telomere-specific reverse transcriptase activity (hTERT), extends telomeres at the end of eukaryotic chromosomes, thus preventing cell senescence and death. Although hTERT is constitutively
present in normal and tumor cells, hTERT is the rate-limiting component of the telomerase complex, and its expression correlates with telomerase activity (11). hTERT activity is repressed in somatic tissues, but both hTERT expression and telomerase activity are elevated in most human tumors (12, 13).

Several studies have showed that hTERT expression and/or telomerase activity were higher in CRC tissue compared with adjacent healthy mucosa (14–20), and some data suggested that telomerase activity increased during cancer progression (21–23). Nonetheless, the prognostic role of hTERT expression is still unclear (20, 23) and its relationship with pathologic stage remains to be assessed. Moreover, a study performed on plasma samples from 50 CRC patients showed that hTERT mRNA levels were higher in patients than in age-matched healthy controls, suggesting that circulating hTERT might be a potential diagnostic marker in CRC (24). However, information on hTERT expression in the corresponding tumors was unavailable and, thus, the relationship between circulating and tumor hTERT transcripts remains to be shown. In addition, it should be emphasized that hTERT mRNA is alternatively spliced in specific patterns in different types of tissues, and such alternate transcripts may constitute a regulatory mechanism for telomerase activity (25, 26). In particular, as α and β regions contain functional reverse transcriptase domains of hTERT, spliced variants lacking these sites do not encode a catalytically active protein (25–27).

The aim of this study was to investigate hTERT expression in CRC at different stages of progression and in corresponding plasma samples by means of an accurate method that quantifies all hTERT transcripts as well as those that selectively encode functional protein.

**Patients and Methods**

**Patients.** The study population consisted of 85 (45 male and 40 female) CRC patients with a median age of 67 y (range, 32-86 y) who underwent surgery at a single institution. None of them received neoadjuvant treatments. Tumors were classified according to the American Joint Committee on Cancer/Union Internationale contre le Cancer tumor-node-metastasis classification and stage groupings (28). The study included 30 patients with stage IV (any T, any N, M1) tumors (median age, 60 y (range, 32-80 y); 16 males and 14 females;...
tumor location: right colon, n = 7; left colon, n = 20; rectum, n = 2; multiple sites, n = 1], 15 patients with stage III (any T, N1-N2, M0) tumors [median age, 72 y (range, 60-86 y); 8 males and 7 females; tumor location: right colon, n = 7; left colon, n = 8], 15 patients with stage II (T3-T4, N0, M0) tumors [median age, 71 y (range, 56-81 y); 8 males and 7 females; tumor location: right colon, n = 9; left colon, n = 4; rectum, n = 2], and 25 patients with stage I (T1-T2, N0, M0) tumors [median age, 69 y (range, 50-84 y); 13 males and 12 females; tumor location, right colon, n = 7; left colon, n = 12; rectum, n = 4; multiple sites, n = 2]. The criterion for selection of tumor samples was the availability of adjacent noncancerous tissue (n = 42) and/or a plasma sample (n = 49). For plasma samples, the control group was composed of 43 healthy subjects (23 males and 20 females) with a median age of 64 y (range, 31-87 y). This was a retrospective study. Biological samples are routinely collected at the time of surgery and stored in the biological bank. At the time of sampling, each patient signed the informed consent approving that his/her biological samples can be used for research purpose. The study was approved by the local Ethical Committee.

Samples. Tissues and plasma specimens were obtained at the time of surgery. From the surgical specimens, samples from both the cancerous lesion and adjacent noncancerous tissue were obtained, immediately shock-frozen in liquid nitrogen, and stored at -80°C until use. Six-micrometer cryostat sections from each tissue sample were prepared using a 1720 Digital cryostat. One section of each sample was stained with H&E for histopathology. All tumor samples analyzed for hTERT expression were from primary tumors and contained >80% tumor cells. RNA was extracted from cryostat sections using 1 mL TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Plasma samples of patients and healthy controls were obtained from peripheral blood by centrifugation on Ficoll-Paque (Pharmacia) and were frozen at -80°C until use. Each plasma sample (500 μL) was separated into 2 aliquots, and each aliquot (250 μL) was mixed with 750 μL of TRIzol LS reagent (Invitrogen). After 5 min of incubation at room temperature, 200 μL chloroform were added to each sample, followed by 15 s of shaking and 10 min incubation at 4°C. The samples were then centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous phase was transferred into a new tube, incubated with 500 μL isopropyl alcohol to allow for RNA precipitation, and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was removed, and the RNA pellet was washed with 1 mL 75% ethanol by centrifugation at 8,000 g for 5 min at 4°C. Thereafter, ethanol was removed, the RNA pellet was dried for 5 to 10 min at room temperature, resuspended in 15 μL of RNase-free water, and then the 2 aliquots were pooled together. The integrity of RNA was evaluated by visualizing the 18S and 28S RNAs through agarose gel electrophoresis, and the relative amount was then quantified by a NanoDrop spectrophotometer (ND-1000; Celbio). RNA was reverse transcribed into cDNA using the SuperScript III RNase reverse transcriptase assay (Invitrogen) in a final volume of 150 μL according to the manufacturer's instructions.

Real-time PCR for quantification of hTERT transcripts. The expression of all hTERT transcripts (hTERT-AT) and the full-length hTERT transcript (hTERT-FL), which encodes the functional protein (25), were quantified by real-time PCR, exactly as previously described (29, 30). In particular, the primers AT1 and AT2 bind to nucleotide (nt) sequences nt 1784 to 1803 and nt 1904 to 1926, respectively, located within the hTERT gene, and allowed detection of all hTERT transcripts, whereas the primers FL1 and FL2 bind to nt 2172 to 2195 and nt 2344 to 2366, respectively, located within the α and β splicing sites, respectively, and thus recognize only mRNA containing the α and β sites and encoding the functional hTERT protein. All real-time PCR reactions were performed using an ABI prism 7900 HT Sequence Detection System (PE Applied Biosystems). Each PCR reaction was performed using 10 μL cDNA sample, and the standard reference curve for quantifying hTERT-AT and hTERT-FL transcripts was performed using serial 5-fold dilutions of the hTERT amplicon, exactly as previously described (29). To normalize hTERT-AT and hTERT-FL copies for the amount of RNA, 10 μL cDNA from each sample was amplified for the housekeeping hypoxantin-guanine phosphoribosyl transferase (HPRT1) gene (31). Amplification was carried out with the forward HPRT1Fw (5’-TCAGCGCACTATAATCCAAAGATGGT3’) and reverse HPRT1Rev (5’-CTGTCTGAGGCTCTTTC3’) primers, which bind to nt 553 to 558 and nt 577 to 597, respectively. The fluorogenic probe (VIC 5’-AAGGTGGCAACAGTT3’ MGBNFQ), which recognizes a sequence located within the amplified product, was synthesized by PE Applied Biosystems. The reference standard curve for HPRT1 was performed using serial dilutions of the HPRT1 amplicon, constructed from cDNA using primers forward HPRT1Fw and reverse HPRT1Rev (5’-TCAGCGCACTATAATCCAAAGATGGT3’). The quantity of all hTERT transcripts was normalized to the copy number of HPRT1. For plasma samples, values of hTERT RNA were also estimated per mL using the conversion factor 15. It should be noted that plasma-derived RNA easily yields fragments amplifiable up to 300 bp (39); the length of PCR products analyzed were all shorter than this length, i.e., 146 and 196 bp for

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Fig. 2. A, levels of hTERT-AT and (B) hTERT-FL transcripts according to tumor stage. Boxes and whiskers. 25th to 75th and 10th to 90th percentiles, respectively; the median is the central line in each box.

B
and specificity are fixed characteristics of a test, the predictive values of value with the highest sensitivity and specificity. Although sensitivity characteristic curve. Using this approach, we selected the optimal cutoff plasma hTERT-AT quantification, we developed a receiver operating correlations were performed with HPRT1/HPRT2 primer pair, the Ct values obtained from samples were then plotted against the calibration weight curve.

Telomerase activity detection assay. Telomerase activity was assessed using the PCR-based telomeric repeats amplification protocol, as previously reported.

Quantification of plasma RNA. To determine the amount of RNA in plasma samples, a standard curve was constructed from RNA stock (TaqMan Control Genome RNA 50 ng/μL; Applied Biosystems). One microgram of RNA was retrotranscribed into cDNA and 5-fold serially diluted in TE buffer [Tris-HCl 10 mmol/L (pH 7.6), EDTA 1 mmol/L (pH 8.0)] from 5,000 to 32 pg/μL. Amplifications were performed with HPRT1/HPRT2 primer pair, the Ct values obtained from samples were then plotted against the calibration weight curve.

Immunohistochemistry. hTERT expression was studied using a specific mouse monoclonal antibody (clone 44F12; Novocastra Laboratories Ltd). Three micrometer-thick sections were cut from formalin-fixed, paraffin-embedded colorectal cancer tissues. Antigen retrieval was performed using microwave heating: the sections were immersed in 10 mmol/L citrate buffer at pH 6 for 4-5 min cycles at 750 W. The slides were then incubated with 10% whole horse serum/PBS for 20 min and then with specific antibody at 1:20 dilution overnight at room temperature. Immunostaining was achieved using the ImmPRESS Anti-Mouse immunoglobulin (peroxidase) kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride chromogen as substrate (Dako). The sections were then lightly counterstained with Mayer’s hematoxylin. Sections of previously tested hTERT-positive neoplastic tissue were processed as positive controls. Negative controls were obtained by replacing the primary antibody with PBS.

Statistical analyses. The distributions of hTERT-AT and hTERT-FL values in tumors and plasma according to tumor stage were compared using the Kruskal-Wallis test. Relationships between tumor and plasma hTERT-AT and hTERT-FL were explored by means of the Spearman rank correlation coefficient. To evaluate the diagnostic performance of plasma hTERT-AT quantification, we developed a receiver operating characteristic curve. Using this approach, we selected the optimal cutoff values with the highest sensitivity and specificity. Although sensitivity and specificity are fixed characteristics of a test, the predictive values of positive and negative tests will vary with the prevalence of the disease. To simulate the diagnostic performance of plasma hTERT-AT, the predictive values of positive and negative test results were estimated based on a prevalence of CRC of 5%. The 95% confidence intervals (CI) were calculated for the correlation coefficients, and the sensitivity and specificity values. All P values were two-sided, and P value of <0.05 was considered statistically significant. Statistical analyses were conducted using R.

Results

hTERT expression correlated with tumor progression. hTERT-AT and hTERT-FL were determined in a total of 85 colorectal tumors. The median level of hTERT-AT was 131 [interquartile range (IQR), 58-324] copies and the median value of hTERT-FL was 18 (IQR, 2-26) copies (Fig. 1A). A significant linear correlation was found between hTERT-AT and hTERT-FL levels (r = 0.849; 95% CI, 0.780-0.900; P < 0.0001; Fig. 1B). Adjacent noncancerous tissue samples were available in 42 cases (17, 3, and 22 from patients with tumor stage I, III, and IV, respectively). Although normal mucosa has been shown to contain hTERT-expressing cells in the proliferative zone of the crypts, patient-by-patient comparisons of matched tissue samples showed significantly lower levels of hTERT transcripts in noncancerous versus cancerous tissue (P < 0.0001). Overall, in noncancerous tissues, the median (IQR) of hTERT-AT and hTERT-FL were 2.5 (0.6-14.7) and 1.2 (0.3-4.7) copies, respectively. In agreement with the results of hTERT mRNA, tumor samples were strongly positive for telomerase activity, whereas adjacent mucosa samples were not (Fig. 1C and D).

Levels of hTERT transcripts were compared with tumor stage. As shown in Fig. 2, both hTERT-AT and hTERT-FL increased with disease progression. Median (IQR) hTERT-AT levels were 42 (26-124), 85 (63-161), 142 (73-260), and 367 (161-1,874).
copies in tumors stage I, II, III, and IV, respectively (overall, \( P < 0.0001 \)); median (IQR) hTERT-FL levels were 2 (1-7), 5 (2-9), 9 (2-14), and 47 (10-125) copies in tumor stage I, II, III, and IV, respectively (overall, \( P < 0.0001 \)). Grading was available for 72 primary tumors: 15 were well-differentiated (grade 1), 48 moderately differentiated (grade 2), and 9 poorly differentiated (grade 3). Median (IQR) hTERT-AT levels were 73 (25-235), 85 (60-474), and 971 (409-3,509) copies in tumor grade 1, 2 and 3, respectively; median (IQR) hTERT-FL levels were 4 (1-14), 5 (2-21), and 63 (11-533) copies in tumors grade 1, 2, and 3, respectively. Well-differentiated and moderately differentiated tumors had significantly lower hTERT-AT (\( P < 0.01 \)) and hTERT-FL (\( P < 0.05 \)) levels than poorly differentiated tumors.

Immunohistochemistry confirmed the nuclear expression of hTERT protein in a large number of cells in stage IV tumors, whereas only a small number of cells expressed a low level of hTERT protein in stage I tumors (Fig. 3). In the adjacent normal colon, hTERT expression was generally absent or confined to the lower half of the crypts. Fibroblasts and inflammatory cells (i.e., lymphocytes and macrophages) intermingling between neoplastic glands were mainly negative for hTERT expression, with only a few lymphocytes very weakly positive (Fig. 3).

**hTERT RNA in plasma correlated with hTERT RNA in tumors.** Plasma samples were available from 49 CRC patients (23 stage I, 2 stage II, 5 stage III, and 19 stage IV) and from 43 age-matched healthy donors. Median (IQR) RNA was 2,306 (1,484-4,583) pg/mL in patients, significantly higher (\( P < 0.0001 \)) than the 22 (0-91) pg/mL obtained from healthy controls. All but two samples from CRC patients were found to be positive for hTERT transcripts with a median (IQR) of 8,183 (2,512-20,065) hTERT-AT copies/mL and 1,474 (542-3,184) hTERT-FL copies/mL. Both patients negative for plasma hTERT-AT RNA had stage I tumors. Similar results were obtained when hTERT levels in plasma were normalized for HPRT1 molecules, i.e., 4,382 (1,593-19,455) hTERT-AT copies/10^3 HPRT1 copies and 934 (220-2,943) hTERT-FL copies/10^3 HPRT1 copies. hTERT-AT correlated with hTERT-FL both when calculated per milliliter (\( r = 0.652; 95\% \text{ CI, } 0.460-0.790; P < 0.0001 \)) and normalized per HPRT1 copies (\( r = 0.722; 95\% \text{ CI, } 0.560-0.830; P < 0.0001 \); Fig. 4).

Conversely, all but three control plasma samples were negative for hTERT mRNA. The three positive samples had 180, 160, and 120 hTERT-AT copies/mL and were from two males, ages 63 and 77 years, and one female, age 63 years; all had negative colonoscopy examination and were without any other neoplasia. From receiver operating characteristic curve...
analysis, the hTERT-AT value, which best discriminated oncologic and healthy subjects, was 180 copies/mL (Fig. 5). By using this cutoff value, sensitivity and specificity of the assay for CRC detection were 92% (95% CI, 80-98%) and 100% (95% CI, 92-100%), respectively. At a 5% prevalence of CRC, this would result in a positive predictive value of 100% and a negative predictive value of 99.6%.

Plasma hTERT levels were significantly lower in patients with stage I and II tumors [median, 2,512 hTERT-AT copies/mL (IQR, 894-5,525) and 884 hTERT-FL copies/mL (IQR, 0-1,932)] than in patients with stage III and IV tumors [median, 19,600 hTERT-AT copies/mL (IQR, 11,030-31,250) and 2,280 hTERT-FL copies/mL (IQR, 1,198-4,483); P < 0.001]. Similar results were obtained when hTERT levels were normalized for 10^3 copies HPRT1, i.e., 1,593 (655-2,783) versus 16,010 (7,222-30,430) hTERT-AT copies and 566 (0-1,018) versus 1,758 (811-3,486) hTERT-FL copies (P < 0.001).

Notably, levels of hTERT-AT in tumors significantly correlated with hTERT-AT levels in plasma both when estimated per mL (r = 0.669; 95% CI, 0.470-0.800; P < 0.0001) and per 10^3 HPRT1 copies (r = 0.702; 95% CI, 0.520-0.820; P < 0.0001; Fig. 6). Tumor and plasma hTERT-FL levels were also significantly correlated (r = 0.532, P < 0.0001 for hTERT-FL/mL; and r = 0.572, P < 0.0001 for hTERT-FL/10^3 HPRT1 copies).

Discussion

Colorectal carcinogenesis is a multistep process, from adenoma to invasive carcinoma. Identification of tumor markers for the detection of CRC in its early stages and by noninvasive tests is an important objective. The inappropriate expression of hTERT in most human tumors makes it a potential diagnostic and prognostic tumor marker. Detection of hTERT transcripts in plasma has also been proposed as a minimally invasive assay for monitoring neoplastic disease (36).

Few studies have addressed the expression of hTERT in colorectal tumors (14–16), and one study has addressed hTERT mRNA in plasma of CRC patients (24). To our knowledge, this is the first study in which hTERT transcripts were quantified in CRC at different stages and in which hTERT levels were determined in paired tumor and plasma samples.

In agreement with several studies addressing telomerase activity in CRC (17, 18, 20, 22), hTERT mRNA levels have been shown to gradually increase from normal mucosa to adenomas and carcinomas (14–16). Nonetheless, hTERT mRNA has also been detected in telomerase-negative samples (23).

Transcripts of hTERT are alternatively spliced (25–27). In particular, mRNA variants lacking α and/or β regions, which contain conserved reverse transcriptase domains of hTERT, do not encode a catalytically active protein. This prompted us to quantify both the levels of all hTERT (hTERT-AT) transcripts and selectively the levels of full-length (hTERT-FL) transcript. Overall, there was a strong relationship between hTERT-AT and hTERT-FL in both tumors and plasma, and the results obtained with hTERT-AT and hTERT-FL were largely superimposable.

hTERT mRNA levels were found to be significantly higher in CRC than in adjacent normal mucosa. Furthermore, hTERT mRNA levels increased with tumor progression, and histologic grade. This finding confirms and expands on previous observations indicating that hTERT mRNA expression was higher in poorly differentiated than in well or moderately differentiated

![Fig. 5.](https://www.aacrjournals.org/click.links/379) Receiver-operating characteristics curve for cancer and healthy patients using plasma hTERT values. Sensitivity (Y axis) was plotted against false-positive fraction (1-Specificity). The area under receiver operating characteristic curve (AUC) is 0.975 (95% CI, 0.919-0.986).

![Fig. 6.](https://www.aacrjournals.org/click.links/380) A, relationship between tumor and plasma levels of hTERT-AT, estimated per milliliter and (B) per 10^3 HPRT1 copies.
tumors (16). Finding that hTERT mRNA was higher in tumors bearing p53 mutations (19) may support the concept that high hTERT expression is a marker of poor outcome and poor response to therapy (29, 37, 38). Notably, positive nuclear staining for hTERT protein in hepatic metastases of CRC was significantly associated with poor prognosis and shorter survival (39). This association between levels of hTERT and tumor progression suggests a potential role of hTERT as a prognostic marker. Further studies including patients with colorectal adenoma will be useful to assess the prognostic value of hTERT.

Transcripts of hTERT were detected in plasma of patients with different tumors. By reverse transcriptase-PCR, hTERT mRNA was detected in 17 of 18 breast cancer tumors and in 4 of 16 available corresponding serum samples (40). Other studies performed using reverse transcriptase-PCR identified hTERT mRNA in serum samples from patients with hepatocellular carcinoma (41), in plasma from patients with lung cancer (42) and gastric cancer (43). Using real-time PCR, hTERT mRNA was recently quantified in patients with prostate cancer. Plasma levels of hTERT mRNA were higher in 68 patients with elevated prostate-specific antigen levels than in 44 controls. Furthermore, patients with prostatectomy patients had lower plasma hTERT mRNA levels than patients with prostate cancer (44).

To date, only one study addressed hTERT RNA in plasma of CRC patients. Real-time PCR analysis of 50 plasma samples from CRC patients revealed that 82% had hTERT mRNA values higher than those observed in plasma of 50 healthy controls. Unfortunately, data on paired tumor samples was not provided in this study; however, sensitivity and specificity of the assay for CRC detection were estimated to be 98% and 64%, respectively (24). Of note, a recent study of gene expression profiling from plasma of 12 CRC patients and 8 healthy subjects did not include hTERT in the list of genes found to be differentially up-regulated in cancer (10). Under-represented probes of the hTERT gene in the microarray likely explain the failure of this approach in detecting differential expression of this gene. In agreement with the study performed using real-time PCR (24), in our study, 47 of 49 (96%) plasma samples from CRC patients were positive for hTERT mRNA. By using a cutoff of 180 copies hTERT-AT/mL, sensitivity and specificity of this assay for CRC detection was 92% and 100%, respectively. It should be pointed out that we evaluated hTERT RNA from plasma samples, thus transcription of hTERT from nonmalignant sources, such as activated lymphocytes, could not be excluded. Furthermore, release of RNA from circulating neoplastic cells may contribute to the plasma levels of hTERT. Notably, levels of hTERT RNA in plasma significantly correlated with levels of hTERT in tumors. Taken together, these results strongly support the concept that hTERT may be a useful biomarker in clinical practice. The close association between tumor and plasma levels of hTERT makes this biomarker a potentially valuable tool in screening studies, and monitoring of neoplastic disease, as well as in the evaluation of response to therapy and tumor recurrence after curative surgery.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Relationship Between Tumor and Plasma Levels of hTERT mRNA in Patients with Colorectal Cancer: Implications for Monitoring of Neoplastic Disease

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