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Advances in Brief

Telomerase Activity Associated with Acquisition of Malignancy in Human Colorectal Cancer[1]

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Abstract

Shortening of telomeres may contribute to the control of the proliferative capacity of normal cells, and telomerase, the enzyme that elongates telomeric DNA, may be essential for unlimited cell proliferation. We have shown previously that telomerase activity is present in human cells immortalized *in vitro* and in metastatic ovarian carcinoma cells but is undetectable in normal cultured cells or normal tissues. We have determined the temporal pattern of telomerase activity during colorectal carcinogenesis in man. We report that telomerase activity is associated with acquisition of malignancy as it is detectable in colorectal carcinoma but not in adenomatous polyps. Mutations leading to reactivation or up-regulation of the enzyme may represent an additional required event in the multistep development of colorectal cancer.

Introduction

The malignant phenotype generally arises from the progressive accumulation of independent mutations that confer to a cell a selective advantage and lead to the expansion of the mutant clone. An excellent paradigm for this multistep process is provided by colorectal cancer, a disease where availability of samples from different stages has allowed detailed characterization of its genetic, histopathological, and clinical course. Somatic mutations in a number of genes appear to be required for the development of a malignant tumor; fewer of the same mutations are involved in benign or premalignant growth (1). The order of appearance of genetic alterations seems less important for tumor progression than does their accumulation (1). However, mutation or loss of the *APC* (adenomatous polyposis coli) gene has been shown to occur early in colorectal carcinogenesis and is indeed a hallmark of the benign tumors (polyps or adenomas) in patients with inherited FAP,[3] a high-risk condition for colon carcinoma (2). As the malignant disease evolves, mutation in the *ras* gene, DNA hypomethylation, and allelic deletion of the *DCC* and *p53* genes are also detected (1). Given the number of mutations leading to colorectal tumors, the successive expansion of mutant clones is likely to involve a total number of cell divisions in excess of the limited proliferative capacity of somatic cells (reviewed in Ref. 3). Thus, in addition to mutations conferring a growth advantage, malignant cells must acquire mutations that override the control of senescence and endow them with unlimited life span (immortality).

Telomere length and telomerase activity have recently been implicated in the control of the proliferative capacity of normal and malignant cells (3). Telomeres correspond to the ends of eukaryotic chromosomes and are composed of specific proteins associated with telomeric DNA, consisting of simple repetitive G-rich sequences (TTAGGG in vertebrates; reviewed in Ref. 4). Telomeres are essential for the stability, identity, and function of chromosomes and provide a buffer against loss of terminal sequences resulting from incomplete replication of linear DNA molecules by unidirectional RNA-primed DNA polymerases (5, 6). Telomeric DNA is synthesized *de novo* by telomerase (reviewed in Ref. 7), a ribonucleoprotein enzyme, which is thus involved in the maintenance of telomere length. Telomeres shorten with replication of human somatic cells in culture and with cell aging *in vivo*, and their length is a good predictor of the replicative capacity of cells (3). It has been proposed that the progressive loss of telomeric DNA, as the molecular event reflecting the number of cell divisions a cell has undergone, would contribute to signaling entry into senescence. Conversely, telomere stabilization by telomerase would abolish such a signal and allow unlimited cell proliferation (3). In agreement with this hypothesis, we have shown that telomerase activity is undetectable in normal or transformed but preimmortal human cells, where telomere shortening occurs, but is present in association with telomere stabilization in immortalized cells (8, 9). Subsequently, we and others have detected telomerase activity in a variety of human tumors and of tumor-derived cell lines (10–13). Taken together, these findings suggest that telomerase activity is required for cell immortality *in vitro* and *in vivo*, and that the enzyme may represent a prevalent or even ubiquitous tumor marker.

Materials and Methods

Telomerase Assay. Extracts were prepared from frozen tissues stored at −80°C by powdering the tissue under liquid nitrogen, followed by addition of 2 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5)-1 mM $MgCl_2$-1 mM EGTA-1 mM phenylmethylsulfonyl fluoride-5 mM β-mercaptoethanol-0.5% CHAPSO-10% glycerol] per mg of powder, incubation for 30 min on ice, with occasional mixing and centrifugation at 100,000 × *g* for 30 min at 4°C. Extracts of 293 cells were prepared by scraping cells from 1 to 2 subconfluent 150-mm dishes, rinsed twice previously with ice-cold PBS lacking calcium and magnesium. Cells were collected by centrifugation, resuspended in ice-cold wash buffer [10 mM HEPES-KOH (pH 7.5)-1.5 mM $MgCl_2$-10 mM KCl-1 mM DTT], pelleted again, resuspended in 15 μl of ice-cold lysis buffer per 10^6 cells, and processed as for tissues extracts. Supernatants were aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the Bradford assay (Bio-Rad). TRAP (12) was used for telomerase assays. Aliquots of extracts, treated or not with RNase to a final concentration of 0.05 mg/ml for 10 min at room temperature, were incubated with 0.1 μg TS oligonucleotide (5′-AATCCGTCGAGCAGAGTT-3′) for 10 min at room temperature in 20 mM Tris-HCl (pH 8.3)-1.5 mM $MgCl_2$-63 mM KCl-0.005% Tween 20-1 mM EGTA-50 μM each dNTP-0.5 mM T4-gene 32 protein-2 μCi [α-^{32}P]dCTP (3000 Ci/mmol), to allow for elongation of the TS primer by telomerase. The reactions were warmed to 92°C before addition of 2 units of Taq polymerase and 0.1 μg of the CX primer (5′-CCCTTACCCT-TACCCTTACCCTAA-3′), and the elongated products were amplified by PCR through 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. During

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; TRAP, Telomeric Repeat Amplification Protocol; TRF, terminal restriction fragment.

Table 1 *Telomerase activity in patients with intestinal diseases*

Patient code	Diagnosis[a]	Age (yr)	Staging[b]	Differentiation[c]	Location	Telomerase activity[d] NM	P	T	NL	LMets
Nonmalignant diseases										
217	DD	46			Colon	–				
274	DD	47			Colon	–				
309	DD	74			Colon	–				
218	CD	52			Colon	–				
316	UC	31			Colon	–				
C1	FAP	38			Rectum	–	–			
C2	FAP	29			Colon	–	–,–			
C3	FAP	23			Colorectum	–	–,–			
C5	FAP	44			Colon		–			
C7	FAP	19			Colon	–	–,–			
C8	FAP	20			Colon	–	–,–			
C9	FAP	41			Colon		–			
C10/11	FAP	60			Duodenum		–			
C12/13	FAP	63			Duodenum		–,–			
C14/15	FAP	55			Duodenum		–,–			
C20	AdP	58			Colon	–	–			
Malignant diseases										
307	ACA	59	T_3N_0	Moderate	Colon	–	–	+		
314	ACA	69	NA	Well/mod.	Colorectum	–	–	+		
C4	ACA	85	T_3N_0	NA	Colon	–		+		
C6	ACA	65	NA	Moderate	Colon	–	–			
008	ACA	74	T_4N_0	Moderate	Colon	–		+		
013	ACA	85	T_4N_X	NA	Colon	–		+		
C17	ACA	80	NA	Moderate	Colon	–		+		
C18	ACA	75	T_3N_1	NA	Colon	–		+		
C19	ACA	65	T_4N_1	NA	Colon	–		+		
C21	ACA	79	T_3N_3	Moderate	Colon		–	–		
C26	ACA	75	T_3N_1	Moderate	Colon	–		+		
C27	ACA	29	NA	Moderate	Rectum	–		+		
C28	ACA	56	T_2N_2	Moderate	Rectum	–		+		
C29	ACA	66	T_3N_0	Moderate	Rectum	–		+		
C30	ACA	46	T_4N_2	Mod./poor	Colon	–		+		
C31	ACA	80	T_2N_0	Moderate	Colon	–		+		
C16	ACA	64			Liver				–	–,–
C22	ACA	58			Liver				–,–	–,–
C23	ACA	62			Liver				–,–	–,–
C24	ACA	70			Liver				+,–	+,+
C25	ACA	50			Liver				–,–	–,–
Total:				No. positive/no. patients		0/26 n = 21	0/15	14/15	1/5[e]	1/5

[a] DD, diverticular disease; CD, Crohn's disease; UC, ulcerative disease; FAP, familial adenomatous polyposis; AdP, adenomatous polyps (sporadic); ACA, adenocarcinoma.
[b] According to the AJCC/UICC classification.
[c] Well/mod, well to moderately differentiated; Mod./poor, moderately to poorly differentiated; NA, not available from tissue report.
[d] All samples were tested at 20 μg protein, except for samples C12/13 and C14/15, which were tested at 5 or 10 μg. Negative samples were retested at 0.2 μg. NM, normal mucosa; P, polyp; T, primary tumor; NL, normal liver; LMets, liver metastases. Positive and negative samples are denoted by + and –, respectively.
[e] For the total, patient C24 was considered positive, although this was the case for only 1 of 2 samples (see text). *n*, no. of polyps.

the course of this study, the assay was improved[4] with the use of end-labeled TS primer and by replacing the CX primer with the ACT oligonucleotide, which is unable to dimerize to TS and anneals at 60°C. The two modifications increased both the specificity and the sensitivity of the assay. Products from one-half of each reaction were resolved in 15% nondenaturing polyacrylamide gels and visualized after exposure to Phosphor-Imager screens. Extracts were considered negative if no telomerase products were detected after a 3-day exposure.

DNA Analysis. TRFs, comprising telomeric and subtelomeric DNA, were obtained from genomic DNA by digestion with restriction enzymes, resolved in 0.5% agarose gels, and visualized by hybridization with a telomeric specific probe, as described previously (8). After exposure to Phosphor-Imager screens (Molecular Dynamics, Sunnyvale, CA), mean TRF length was calculated from the total counts between 21 and 2 kbp, determined with the use of ImageQuant software (Molecular Dynamics).

Results and Discussion

Previous studies showing reduced telomere length in a high percentage of adenocarcinoma relative to normal colorectal mucosa but comparable loss of telomeric DNA in benign tumors (polyps or adenoma) and in carcinomas (14, 15) suggested that activation of telomerase during colorectal carcinogenesis, if it occurs at all, may take place at either the benign or malignant stage. To establish the temporal pattern of enzyme expression and to determine whether presence of telomerase could have diagnostic or prognostic value, we have assayed enzymatic activity in surgically excised samples from different grade colorectal tumors and control tissue.

Tissue samples were obtained at resection from 37 patients (Table 1) at the Mount Sinai Hospital (Toronto) or the Henderson Hospital (Hamilton). Five of these patients presented with inflammatory diseases (diverticular disease, ulcerative colitis, or Crohn's disease). The remainder was patients with familial adenomatous polyposis (10 individuals), sporadic colorectal polyps (1 individual), colorectal adenocarcinoma (16 individuals), or liver metastases from previously resected colorectal adenocarcinoma (5 individuals). Samples from 3 of the FAP patients were obtained from duodenal periampullary polyps, a common, extracolonic, premalignant feature of the disease (16). The age of individuals ranged from 19 to 85 years, with overlap between the ages of cancer and noncancer patients (Table 1). Protein extracts were prepared by detergent lysis (12) from intestinal mucosa of patients with diverticular disease, ulcerative colitis, or Crohn's disease; from histologically normal mucosa adjacent to cancerous tissue; and from polyps and carcinoma tissues.

[4] N-W. Kim, personal communication.

Telomerase activity was assayed with the use of the highly sensitive TRAP assay (12) capable of detecting activity in as few as 10 positive cells or as little as 0.01% positive cells in a mixed population. Enzymatic activity was detected as a 6-nucleotide repeat ladder that was sensitive to pretreatment of the extracts with RNase, as shown in Fig. 1*A* for the immortal human 293 cell line, our positive control. Initial assays were performed at 30 PCR amplification cycles and 20 μg of protein, except in the case of 3 very small polyps that were assayed at 5 or 10 μg protein (Table 1). Because dilution of highly positive extracts, such as those from 293 cells, was found to enhance the level of enzymatic activity (Fig. 1*A*), telomerase-negative tissue extracts were reassayed at 0.2 μg protein. Inhibition of telomerase activity at high protein concentrations appears to be characteristic for the PCR-based TRAP assay because activity of the 293 cell line was found to be proportional to protein amounts ranging from 2 to 200 μg with the use of the conventional assay (10).

Representative results of telomerase assays of tissue samples are shown in Fig. 1*B*, and results for all patient samples are summarized in Table 1. Enzyme activity was not detected in any sample of histologically normal mucosa, whether from cancer or noncancer patients. Similarly, all of the 21 polyps from 15 different patients were found to be telomerase negative. Lack of enzymatic activity in negative extracts was confirmed in all cases by assaying at 100-fold lower protein concentration. In contrast, enzymatic activity was detected in 14 of 15 adenocarcinomas. Activity in these samples was detectable only by assaying 20 μg protein and was lower than that of 293 cells, except for 1 case in which activity was comparable to that of 293 cells and was enhanced by dilution of the extract (not shown). Lack of telomerase activity in the single negative sample of adenocarcinoma was confirmed by assaying a different section of the biopsy and again by dilutions of the extracts. Moreover, although this sample was about 20% necrotic, it had similar levels of DNA polymerase activity as telomerase-positive tumors (data not shown), and it did not differ from them in other clinical characteristics or in age of the donor. It remains possible that lack of telomerase activity may be related to degradation of the essential telomerase-templating RNA; however, undetectable telomerase activity has been reported by others in about 10% of tumor samples (12), a finding that may suggest foreshortened tumor development due to acquisition of hypermutability by some cells or alternative pathways to telomere maintenance.

Telomerase activity was not detected in histologically normal liver from 4 patients presenting with liver metastases from a previously resected colonic tumor. However, one of two biopsies from a fifth patient was positive, perhaps due to the presence of micrometastases. Two independent samples of liver metastases from 5 patients were also negative, except for a single patient (Table 1). These results were unexpected given the extremely high rate of positivity of primary tumors (14 of 15) and the fact that 7 of 7 metastatic samples of ovarian carcinoma were found to be telomerase positive (10). Because liver abounds in degradative enzymes and, moreover, is devascularized during surgery for a significantly longer period of time than is colon (2–3 h *versus* 20–30 min), we assayed for another replicative enzyme. Three of the four telomerase-negative extracts had no detectable DNA polymerase activity, and in the fourth the levels of this enzyme were about 6-fold lower than in positive tumors. Even the single telomerase-positive metastasis had reduced DNA polymerase activity but only by about 3-fold (data not shown). These results are compatible with generalized protein degradation as the cause for the lack of detectable telomerase activity in liver metastases. They also provide a cautionary note for the negativity of histologically normal liver samples in this or similar studies.

TRFs of variable length relative to normal colorectal mucosa have been detected in adenocarcinoma (14, 15). In 3 adenomatous polyps included in 1 study (14), TRFs were shorter than in isogenic normal tissue and comparable to those of the matching tumor. We have measured the length of TRFs in a matched series of normal mucosa and 11 polyps from 8 patients (not shown). As for adenocarcinoma (14, 15), we found that TRFs in polyps varied in length [shorter (5 of 11), comparable (2 of 11), or longer (4 of 11) than in control tissue]. The latter polyps were from 2 patients with unexpectedly short TRFs in normal mucosa for their age. Variability in TRFs of polyps is likely to reflect clonal variation because all samples were negative for telomerase, and polyp size was not sufficiently different to account for differences in the length of TRFs.

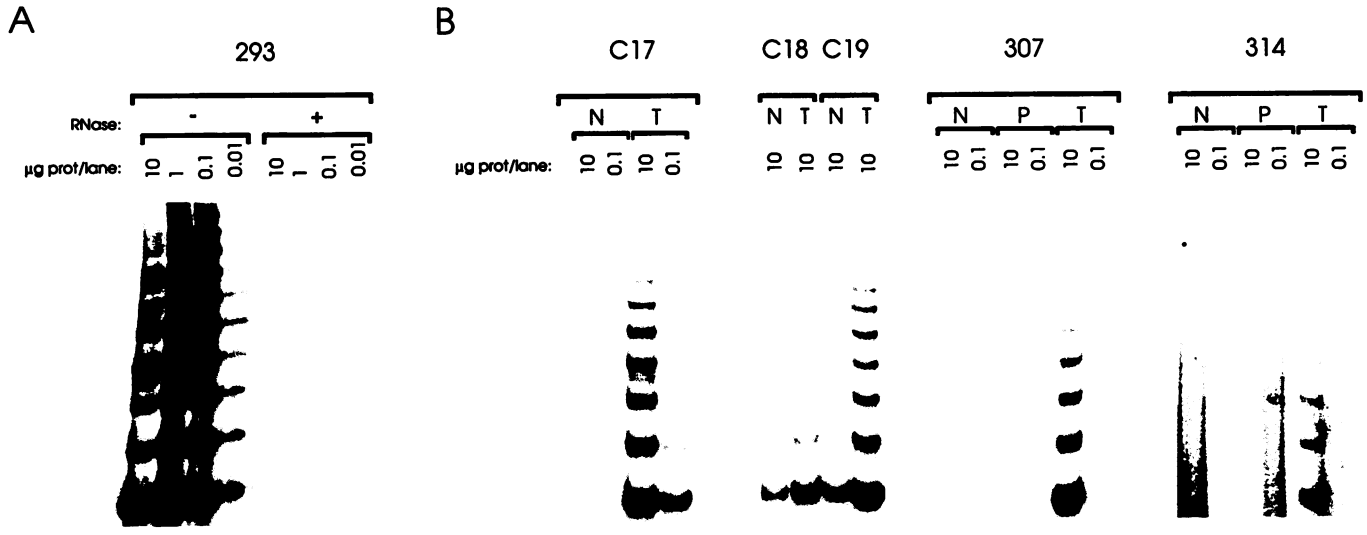

Fig. 1. Telomerase activity in control and malignant tissues. Enzyme assays were performed by the TRAP method with the use of the ACT second primer, except for C18 and C19, where the CX primer was used. Reaction products were resolved on nondenaturing gels and visualized by Phosphor-Imager analysis, with the use of limits of sensitivity of 5000/50000 counts for the most active samples (293 and C17), 1000/10000 counts for samples with intermediate activity (C18, C19, and 307), and 500/1500 counts for the least active sample (314). Amounts of protein (*prot*) are indicated above each lane. All extracts were assayed with (+) or without (−) pretreatment with RNase, but results of RNase digestion are shown only for the positive control, the 293 cell line (*A*). Representative assays of adjacent normal mucosas (*N*), polyps (*P*), and tumors (*T*) from 5 patients (C17, C18, C19, 307, and 314) are shown in *B*. The single band in normal mucosa samples from patients C18 and C19 is likely due to primer-dimer formation between the TS and CX primers (see "Materials and Methods").

The aim of our study was to determine the temporal pattern of telomerase activation during colorectal carcinogenesis. We have shown that detectable telomerase activity correlates with acquisition of malignancy from benign adenomatous polyps to invasive colorectal cancer. Similar conclusions from a survey of 8 colorectal carcinomas and 2 polyps were reported recently (12). Although we did not detect telomerase activity in 21 polyps from 15 patients, consistent with lack of the enzyme in the precancerous stage, positivity in a few polyps would not be surprising because about 5% of colorectal adenomas may contain malignant foci (17). As a specific marker for malignancy in colorectal and other forms of cancer (10–13), telomerase may represent an excellent target for cancer therapy. Moreover, it may be potentially exploited, in combination with other approaches, as preventive therapy for patients presenting with FAP or other high-risk syndromes of colorectal cancer. At present, prophylactic colectomy is the only effective therapy available to these patients, although the possibility of replacing surgery with chemotherapy is currently being investigated (18). In recent work on mammary tumorigenesis,[5] we concluded that transgenic mouse lines represent suitable animal models for assessing the outcome of telomerase inhibition on malignant and normal tissues. The *Min* (19) and *APC*1638 (20) mice, carrying mutations in the *APC* gene, develop multiple intestinal tumors and are potentially useful models of human FAP. These mouse lines should prove valuable for investigating approaches to telomerase inhibition for the prevention of colorectal carcinogenesis.

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