Telomerase as a diagnostic and predictive marker in colorectal carcinoma*

J. MALÁSKA[1,2], Z. KUNICKÁ[3], M. BORSKÝ[4], M. SKLENIČKOVÁ[1], M. NOVOTNÁ[3], L. FAJKUSOVÁ[3,4], J. ŽALOUDÍK[2], J. FAJKUS[1,3**]

[1]Institute of Biophysics, Academy of Sciences of the Czech Republic, 61265 Brno, e-mail: fajkus@ibp.cz, [2]Masaryk Memorial Cancer Institute, Brno, [3]Department of Functional Genomics and Proteomics, Masaryk University Brno, [4]University Hospital Brno, Department of Internal Medicine and Hematooncology, Centre of Molecular Biology and Gene Therapy, Brno, Czech Republic

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In a search for molecular markers providing both informative diagnostics of malignant disease, and rational stratification of a therapeutic strategy to achieve optimal response in a given patient, we examined the possibility of using telomerase for this purpose in colorectal cancer. Telomerase, a ribonucleoprotein enzyme complex catalysing synthesis of chromosome ends (telomeres), has been known as an almost universal tumor marker but its predictive value has been found in only a limited number of malignant tumor types. Telomerase activity and expression of its catalytic subunit hTERT was determined in 82 surgical specimens from 41 patients (a sample of tumor tissue and of adjacent morphologically normal tissue was obtained from each patient). Telomerase activity was present in tumor samples from 34 (83%) patients, reaching an average value of 47.6 telomerase units (T.U.), while adjacent tissue specimens were either negative (in 25 (61%) patients), or slightly positive (in 16 (39%) patients) showing 1.5 T.U. on average. In tumor samples from patients without lymphatic node metastases (pN0), an average of 37.1 T.U was found. In contrast, in tumor samples from patients with lymphatic node involvement (pN1 or pN2) the average activity was significantly higher (60.2 T.U., $p<0.05$). In patients with distant metastases a tendency towards higher telomerase activity, although lacking statistical significance, could be observed. Among patients that obtained chemotherapy with 5-fluoruracil, those with low telomerase activity showed a tendency to chemosensitivity. Expression of hTERT was detected not only in samples showing telomerase activity, but also in a considerable portion of telomerase-negative samples either from the tumor or the adjacent normal tissue. We demonstrate that some of these apparent discrepancies may be attributed to differential splicing of hTERT mRNA. We conclude that TRAP assay for telomerase activity is more informative than the common testing for hTERT expression. Telomerase activity is useful both as a diagnostic as well as a predictive factor in colorectal cancer.

Key words: colorectal carcinoma, telomerase, diagnostic and predictive marker

Colorectal carcinoma belongs to the group of most frequently diagnosed tumors and is a significant cause of morbidity and mortality in all over the world. Its increasing incidence and the relatively low efficiency of chemotherapy represent major therapeutic problems. Besides the development of new cytostatic drugs, another approach to therapeutic progress could arise from individual planning of a therapeutic strategy based on evaluation of predictive factors, i.e. molecular determinants allowing to assess the effect of different modalities of anticancer therapy. A number of genetic alterations accompanies the pathologic transformation of normal colonic epithelium to an adenomatous polyp and ultimately an invasive cancer. It is therefore feasible to search for such markers among genes whose mutations are involved in this long-term multistep process of disease progression. These include for example adenomatous polyposis coli (APC), K-ras protooncogene, DCC (Deleted in Colorectal Cancer) tumor-suppressor gene, tumor-suppressor genes of the transforming growth factor beta (TGF-beta) pathway, p53 tumor-suppressor gene and genes involved in DNA mismatch repair [8].

Besides gene mutations and possibly other (epigenetic)

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**Author to whom correspondence should be sent.

Table 1. An overview of previous results on the possible predictive value of telomerase activity in colorectal cancer

No. of tumor specimens tested	Presence of telomerase activity	Tested correlation(s) of telomerase activity with other clinicopathogenic factors / result	Reference
23	23 (100%)	clinical staging / no; p53 mutations / no	[2]
37	19 (51%)	Astler and Collier staging / yes (C>A,B) lymph node metastasis / yes differentiation grading / yes (poorly and moderately > well) mitotic and Ki-67 labeling index / yes Bcl-2 expression / no p53 accumulation / no	[10]
67	50 (75%)	microsatellite instability / no	[16]
29	29 (100%)	Dukes' staging / yes (A and C>B) location / yes (right>left) differentiation grading / yes (poorly>well)	[7]
50	33 (66%)	clinical staging / no Bcl-2 expression / yes (higher activity in Bcl-2-positive samples)	[9]
30	27 (90%)	depth of invasion / yes age, gender, histologic type, location / no lymph node metastasis, lymphatic infiltration, Dukes' stage / no venous invasion / yes liver metastasis / yes	[13]
20	15 (75%)	depth of tumor invasion / no lymphatic and/or venous involvement / no regional lymph node metastasis and Dukes' stage / no	[1]

changes leading to altered gene expression (e.g., DNA methylation and histone acetylation), transformed cells have to possess a functioning telomere-maintenance mechanism to escape cell cycle arrest or apoptosis after critical telomere shortening due to incomplete chromosome-end replication. In about 85% of all cancers and about the same percentage of colorectal cancers [11], telomere maintenance is achieved by activation of telomerase, a complex synthesising telomeres via the reverse transcriptase activity of its catalytic protein subunit, hTERT, using a template region of an RNA subunit, hTR (see [6] for recent review). The rest can be accounted for by alternative telomere lengthening (ALT) mechanisms operating via a telomerase-independent (probably recombination) pathway [3] . Consequently, telomerase activity has become an almost universal diagnostic marker in oncology, but its predictive value has been shown in only a limited number of tumor types. In colorectal cancer, there are discrepant reports on this subject. A significant correlation of telomerase activity with tumor staging, cell differentiation, proliferation and lymph node metastasis in colorectal carcinomas has been reported [10]. Another group observed a significant difference in telomerase activity between tumors with and without venous invasion [13]. Telomerase activity was correlated with depth of invasion and liver metastasis but not with lymph node metastasis, lymphatic infiltration or Dukes stage. In another recent study [1] no significant correlation was observed between telomerase activity levels and clinicopathological parameters such as depth of tumor invasion, lymphatic and/or venous involvement, and regional lymph node metastasis and Dukes' stage. An overview of previous results is given in Table 1.

The aim of our present study was therefore to bring independent novel data to the ongoing discussion on the diagnostic and predictive value of telomerase in colorectal cancer. These data show not only a strong correlation of telomerase activity with malignant phenotype, but also with lymph node metastasis. We further show that assays for telomerase activity are more informative than a simple RT-PCR test of hTERT expression, as the latter is also positive in a considerable portion of telomerase-negative samples coming either from the tumor or the adjacent normal tissue. We also demonstrate that some of these apparent discrepancies may be attributed to differential splicing of hTERT mRNA [4, 14, 17].

Material and methods

Patients. Surgical specimens of tumor and adjacent mor-

phologically normal tissue were obtained from 41 patients who provided written informed consent to provide samples for molecular genetic examination (a total of 82 specimens were analyzed). Diagnosis of colorectal adenocarcinoma was confirmed by histological typing. Among 41 patients, 3 showed grade I., 34 grade II. and 3 were of grade III., and in one sample this criteria could not be applied. In the TNM classification, there were 3 of pT2, 20 of pT3 and 17 of pT4 stage, and in one sample this was not applicable. With respect to lymphatic node involvement, there were 24 patients of pN0, 12 of pN1 and 2 of pN2 classification, and in 3 samples the pN status could not be assessed (see also Table 2 in the Results section).

Telomerase activity assay. A TRAPeze Telomerase Detection Kit (Intergene) was used for preparation of tissue extracts from surgical specimens and for semiquantitative TRAP assays according to the supplier's instructions. Two reactions using aliquots corresponding to tumor tissue and adjacent tissue were tested in parallel. Reaction products were separated on 12.5% polyacrylamide gels (19:1 acrylamide:bisacrylamide), stained using SYBR Green I (Molecular Probes) and detected in blue fluorescence mode on a PhosphorImager STORM860 (Molecular Dynamics). Semi-quantitative evaluation in relative telomerase units (T.U.) was performed using ImageQuant software (Molecular Dynamics).

RNA isolation, RT-PCR analysis of hTERT expression and detection of differential hTERT splicing. Total RNA was isolated from surgical specimens using a RNeasy Mini Kit (QIAGEN) and the quality of the product was checked on an agarose gel. RNA concentration was measured using RiboGreen RNA quantitation reagent (Molecular Probes). Random hexamer-primed cDNA was synthesized from 200 ng of total RNA in a 20 μl reaction using a GeneAmp RNA PCR kit (Applied Biosystems). Amplifications were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) in the supplied reaction buffer and 2 mM $MgCl_2$ with an initial denaturation at 95 °C for 10 min followed by 40 cycles (95 °C/30 s, 55 °C/30 s, 72 °C/30 s) and a final extension (72 °C/7 min). As an independent internal marker of overall transcription, expression of HPRT was detected by RT-PCR in parallel.

For analysis of differential splicing of hTERT mRNA, cDNA was prepared using 200 ng of RNA and a Super Script First Strand Synthesis System for RT-PCR (Gibco BRL) with random hexamers. The amplification of alternatively spliced hTERT cDNA was performed with TERT-2164S and TERT-2620A primers as described [15]. Products were labeled during PCR with α-[^{32}P]dCTP (75 kBq per PCR reaction). Amplified products were electrophoresed on 6% polyacrylamide gel with 1% urea and visualized by use of PhosphorImager or X-ray film. As an internal control, expression of HPRT was detected by RT-PCR in parallel.

Results and discussion

The overview of results is given in Table 2. In these data, telomerase activity is positive in 34 of 41 tumor specimens (83%) showing an average of about 48 telomerase units (T.U.). This percentage corresponds well with summarized data obtained by other groups [12]. Tumor-adjacent tissue specimens are either negative (in 25 (61%) patients), or slightly positive (in 16 (39%) patients) showing 1.5 T.U. on average. Expression of hTERT was detected by RT-PCR in all 34 telomerase-positive tumor specimens and in 3 of 7 telomerase-negative tumor specimens. This apparent discrepancy can be explained in all these 3 samples by the presence of an α^- splicing variant of hTERT mRNA whose inhibitory effect has been reported recently [4, 17] or, alternatively, by the absence of the full-length ($\alpha^+\beta^+$) hTERT mRNA transcript (Fig. 1). To our knowledge, our data represent the first report on alternate hTERT mRNA splicing in colorectal cancer specimens and correspond to our previous findings in normal and carcinoma colon cell lines [5].

Expression of hTERT was found also in 19 specimens from adjacent morphologically normal tissue, mostly in specimens where a slight telomerase activity had been detected. The slight activity and positive RT-PCR results can be explained in these cases by invasion of tumor cells to adjacent tissue and/or by presence of normal proliferating cells in colon crypts.

To test the latter possibility, additional control specimens from 3 non-tumor patients (samples of colorectal mucosa

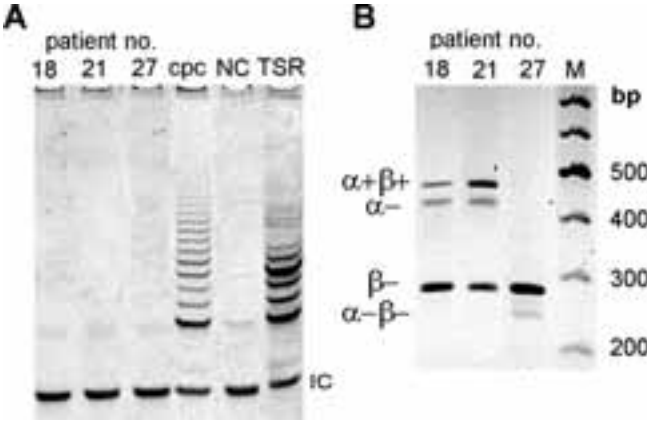

Figure 1. Analysis of telomerase activity (A), expression and splicing pattern (B) of the hTERT mRNA region 2164-2620 (Genebank AF15950) in selected tumor samples. Tumor samples of patients 18, 21 and 27 display no telomerase activity in TRAP assays (A), although they show expression of hTERT mRNA (B). This apparent discrepancy can be explained by the presence of an inhibitory α^- splicing variant of hTERT mRNA (patient 18 and 21) or by the absence of the full-length length ($\alpha^+\beta^+$) hTERT mRNA transcript (patient 27).
cpc – control positive HeLa cells, NC – negative control, TSR – telomerase quantitation control, IC – internal amplification control, M – DNA length marker.

and of complete colon section) were analyzed in parallel. No hTERT mRNA expression was detected in all 3 samples of colorectal mucosa, while it could be found (showing the same splicing pattern) in complete colon sections, probably reflecting the presence of proliferating cells from colon crypts. Considering these results we conclude that testing of hTERT mRNA expression is less informative than TRAP assays for telomerase activity, unless a quantitative version of RT-PCR and complementary information on the splicing pattern are available.

Concerning the potential predictive value of telomerase activity, tumor samples from patients without lymphatic node metastases (pN0) show an average value of 37.1 T.U. In contrast, in tumor samples from patients with lymphatic node involvement (pN1 or pN2) the average activity was significantly higher (60.2 T.U., $p<0.05$). These data thus correspond to previous findings showing a correlation of telomerase activity with lymph node metastasis [10], but contradict a couple of other reports [1, 13].

In patients with distant metastases a tendency towards higher telomerase activity, although lacking statistical significance, could be observed. Among twelve patients that obtained chemotherapy with 5-fluoruracil, eight had objective response. Six of these patients showed low levels of telomerase activity (0.0–6.0 T.U.) while within the non-responding group only one patient with no activity occurred.

We conclude that telomerase activity is useful both as a diagnostic and a predictive factor in colorectal cancer.

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