Expression of the Human Telomerase Reverse Transcriptase Is Not Related to Telomerase Activity in Normal and Malignant Renal Tissue

Volker Rohde, Hans-Peter Sattler, Thorsten Bund, Helmut Bonkhoff, Thomas Fixemer, Christa Bachmann, Ramona Lensch, Gerhard Unteregger, Michael Stoeckle, and Bernd Wullich

ABSTRACT

In this study, the association between telomerase activity and the expression of the human telomerase subunits human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) in paired neoplastic and normal renal tissue samples was investigated. Reverse transcription (RT)-PCR on 20 tumor nephrectomy samples revealed that hTR was constitutively expressed both in cancer and normal tissue samples, independent of the telomerase activity status. Remarkably, using in situ hybridization, the expression levels of hTR were found to be markedly higher in the normal tissue than those in the tumors. Expression of hTERT mRNA by RT-PCR was observed in 90% of the cancer samples and, notably, also in 75% of the corresponding normal renal tissue samples. Because all of the normal tissue samples and some of the tumor samples were shown to be telomerase negative, our findings suggest that hTERT mRNA expression is not sufficient for telomerase enzyme activation. Furthermore, semiquantitative RT-PCR revealed equal or even higher hTERT mRNA expression levels in the telomerase-negative normal samples than in the corresponding cancer samples with telomerase activity, contradicting the assumption that a certain threshold level of hTERT mRNA is required for telomerase activation at least in renal tissue. It seems more likely, that other mechanisms, such as posttranscriptional modification of hTERT or inactivation of telomerase inhibitors, are involved in the acquisition of enzyme activity.

INTRODUCTION

Telomeres form the distal ends of eukaryotic chromosomes consisting of several thousand copies of hexameric TTAGGG repeats. These noncoding sequences are thought to be important to protect the coding regions of the DNA from degradation and other genetic changes (1). In the life span of a somatic cell, the telomeric DNA shortens ~50–100 bp with each cell division, because the 5' end of linear DNA cannot be fully replicated, a fact that is known as the “end replication problem” (2). The telomere hypothesis of cellular senescence postulates that the progressive shortening of the chromosome ends in somatic cells results in cell cycle exit (3). In contrast to somatic cells, germ cell lines do not show substantial loss of telomeric repeats with increased age because of the activation of the ribonucleoprotein telomerase (4). Telomerase adds telomeric repeats to the telomeric DNA depending on its RNA component hTR4, which serves as a template. The activity of this enzyme is also detectable in many human tumors and tumor-derived cell lines (5). The activation of telomerase seems to be concomitant with the attainment of immortality, which is assumed to be critical in sustaining malignant tumor growth (6).

Recently, the gene of the putative catalytic subunit of the human telomerase hTERT [previously referred to as hTRT (7), hEST2 (8), and hTCS1 (9)] was cloned. Its expression at the mRNA level has been reported to be strongly associated with enzyme activity and concomitant immortalization. Its introduction into normal human epithelial cells and fibroblasts was sufficient to reconstitute telomerase activity, arrest telomere shortening, and extend the life span in vitro (7, 8, 10–12).

In several studies on human renal cell carcinomas, telomerase activity has been found in 56–83% of the tumors analyzed (13–19). We recently reported detection of telomerase activity not only in cancer lesions but also in a small fraction of histologically normal parenchyma from cancer-bearing kidneys (20). Because there is increasing evidence that telomerase, aside from germ cells, can also be activated in other nonneoplastic cells, such as proliferating cells of renewal tissues and activated lymphocytes (21–27), the question arises of the identity of the telomerase-expressing cells within a heterogeneous biopsy.

In the present study, we set out to analyze the association...
between telomerase activity and the expression of hTR and hTERT mRNA in normal and neoplastic renal tissue. RT-PCR analyses were furthered by in situ hybridization to study hTR expression at the single-cell level in normal and cancer renal tissue.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Tumor specimens were obtained from 20 patients who underwent nephrectomy for renal cell carcinoma. For comparison, corresponding normal renal tissue samples were taken in each case. All of the samples were obtained immediately after kidney removal in the operation theater. Each sample was divided into two parts that were snap frozen in liquid nitrogen and stored at −80°C until protein or RNA extraction was performed. The histological diagnosis was conducted on sections from the same samples that were used for the telomerase assay and RNA expression studies. To minimize sampling error, control sections from the top as well as from the bottom of each sample were evaluated using standard staining protocols. This was done in addition to routine histopathological examination of the nephrectomy specimens, which were classified according to the TNM classification system, 5th revision (28). Preoperative examination of the patients, including pulmonary X-ray, ultrasound, and computerized tomography, revealed no distant metastasis. None of the patients had been treated with radiation, chemotherapy, or immunotherapy before nephrectomy. For control, tissue samples from two hydronephrotic kidneys without malignancy were assayed for telomerase activity and expression of the telomerase subunits.

The immortal prostatic carcinoma cell lines PC-3 and DU145 were obtained from the American Type Culture Collection. From subconfluent cultures, cells were harvested by trypsinization and washed with PBS, and pellets were resuspended in TRAP lysis buffer or TriZol (Life Technologies, Eggenstein, Germany) for extraction of protein or RNA, respectively.

TRAP Assay. Preparation of the tissue extracts and the TRAP assay were performed as described elsewhere (20). Briefly, frozen tissue samples were homogenized in 200 µl ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 12 mM EGTA, 0.1 mM 4(2-amino)benzesulfonyl fluoride hydrochloride, 0.5% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), and 10% glycerol], incubated on ice for 30 min, and centrifuged at 16,000 × g for 30 min at 4°C. The supernatant was rapidly frozen and stored at −80°C.

For the TRAP assay, 2 µl of the tissue extract (6 µg protein equivalent) were added to 46.5 µl of the reaction mixture [50 µM dNTPs, 0.5 µM T4gene32 protein (Roche Diagnostics, Mannheim, Germany)], 5 µl TRAP assay buffer (20 mM Tris-HCl, 1.5 mM MgCl₂, 62 mM KCl, 0.005% Tween 20, and 1 mM EGFA), 0.1 mg/ml BSA, and 0.1 µg TS primer. The reaction solution was layered with mineral oil and incubated for 30 min at room temperature, mediating extension of the TS primer. The oligonucleotides used (TS and CX primer) were previously described (29). Afterward, they were heated to 90°C, and 0.1 µg CX primer and 2.5 units Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) were added to each tube. Forty PCR cycles were run under the following conditions: 94°C for 20 s, 55°C for 20 s, 72°C for 30 s. Aliquots of the PCR products were analyzed by electrophoresis on a 15% nondenaturing polyacrylamide gel. The 6-bp ladder units were detected by silver staining.

As a positive control, 2 µl of a protein extract from the cell line PC-3 with known telomerase activity were used. As a negative control, 2 µl lysis buffer were assayed in each experiment. All of the extracts showing 6-bp ladders were tested for sensitivity to RNase A pretreatment. Only samples that produced RNase-sensitive ladders extending four or more units were considered positive for telomerase activity. Positive results were confirmed by repeat experiments. To exclude the presence of Taq polymerase inhibitors in the tissue extracts of the telomerase-negative samples, they were checked by another TRAP assay, including a 150-bp internal DNA standard (30).

RT-PCR. Total RNA was isolated from frozen tissue using TriZol, which was DNase-digested and reverse-transcribed with a RT-PCR kit (Stratagene, La Jolla, CA). The resulting cDNA was subjected to PCR using the following primers and PCR conditions: hTR: 5’TCT AGA CTC CAT GGG GAA GGT GAA-3’ and 5’GGC GAA CGG GCC AGC AGC TGA CAT T-3’ (TRC3F; Ref. 31); PCR conditions were as follows: initial incubation at 94°C for 5 min, 36 cycles with 94°C for 20 s, 55°C for 20 s, 72°C for 20 s, and a final incubation at 72°C for 10 min. hTERT mRNA was amplified using the primer pair 5’-CGG AAG AGT GTG TGC AGC AA-3’ (LT5) and 5’-GGA TGA AGC GGA GTC GTG A-3’ (LT6; Ref. 7); PCR conditions were as follows: initial incubation at 94°C for 5 min, 34 cycles with 94°C for 20 s, 60°C for 20 s, 72°C for 30 s, and a final incubation at 72°C for 10 min. For estimating both proper RNA quality and successful cDNA synthesis from each sample, PCR with GAPDH-specific primers of 5’-CTC AGA CAC CAT GGG GAA GGT GA-3’ (K136) and 5’-ATG ATC TTC AGG CTA TG TGAACA-3’ (K137) was performed; PCR conditions were as follows: initial incubation at 94°C for 5 min, 30 cycles with 94°C for 45 s, 55°C for 45 s, 72°C for 1 min 30 s, and a final incubation at 72°C for 10 min.

PCR products were separated on a 2% agarose gel and visualized by SYBR-Green I staining (BIOzym, Hes. Oldendorf, Germany), which increases sensitivity compared with that of ethidium bromide. In each experiment, the telomerase-positive PC-3 cell line served as a positive control and a RNase A-digested template as a negative control.

For semiquantitative assessment of hTERT mRNA levels, the procedure given by Snijders et al. (32) was applied with modifications. Cancer and corresponding normal renal tissue samples as well as 100 ng DU145 RNA were subjected to another RT-PCR round for 30 cycles. At this number of PCR cycles the amplification reaction showed linearity, as was determined on three different dilutions of RNA input from DU145 and tissue samples. All of the PCR products were run on the same agarose gel and visualized by SYBR-Green I staining. Densitometric evaluation of the signal intensities was performed using the program Gelscan 3D, Version 2.0 (Science Group, BioSciTec, Marburg, Germany). Levels of hTERT mRNA, normalized to the hTERT levels from 100 ng DU145 RNA, were calculated according to the following formula: (intensity ratio...
hTERT-GAPDH of the sample): (intensity ratio hTERT-GAPDH of 100 ng DU145 RNA).

hTR in Situ Hybridization. For hTR detection, 5′ fluorescent-labeled oligonucleotides with the following sequences were used: 5′-GGT GCC CAT TTT TTG TCT AAC CCT AAC TGA GAA GCC CGT AGG CGC CG-3′ (antisense configuration); 5′-CGG CCG CTA CCG CCT TCT CAG TTA GGG TTA GAC AAA AAA TGG CCA CC-3′ (sense configuration). The sequences were given by Sallinen et al. (33). The sense probe served as negative control. Formalin-fixed, paraffin-embedded tissue sections (5-μm thickness) were deparaffinized, and after digestion in 400 μg/ml protease K (Merck, Darmstadt, Germany) at 37°C for 15 min, the sections were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. After they were washed twice in 2× SSC, the slides were covered with 40 μl of the prehybridization buffer containing 2× SSC, 1× Denhardt’s solution (50× Denhardt’s stock solution: 1% polyvinylchloride, 1% pyrrolidone, 2% BSA; Oncor, Heidelberg, Germany), 10% dextran sulfate (Roche Diagnostics), 50 mM phosphate buffer (pH 7.0; Merck), 50 mM DTT (Roche Diagnostics), 250 μg/ml yeast tRNA (Roche Diagnostics), 100 μg/ml polyadenylic acid (Roche Diagnostics), 500 μg/ml denatured and sheared DNA from fish sperm (Roche Diagnostics), and 26.7% deionized formamide (Oncor) and were incubated for 2 h at 37°C in a humid chamber. The sections were hybridized overnight at 37°C with 40 μl of hybridization mixture containing the hybridization buffer and 10 pmol labeled oligonucleotide per slide. After washing in graded concentrations of SSC (2×, 1×, 0.25×) at 37°C for 30 min, the sections were incubated with a mouse monoclonal antifluorescin antibody, followed by a biotinylated antimouse antibody and HRP-labeled avidin-biotin complex method (ABC-HRP; Dako, Hamburg, Germany). A signal amplification method based on the deposition of biotinylated tyramine was used to enhance immunodetection. After precipitation of the biotinylated tyramine (10 min at room temperature) through the enzymatic action of HRP and H2O2 (0.1%), the biotin precipitate was detected with an additional application of the HRP-labeled avidin-biotin complex for 30 min in a humid chamber. The peroxidase reaction was developed by 3,3′-diaminobenzidine (Sigma, Deisenhofen, Germany).

In each experiment, a RNase A-digested slide was used as a negative control. The slides were digested for 5 min at 55°C in a digestion buffer [0.5 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.2)] containing 10 μg/μl RNase A (Roche Diagnostics). Only RNase-sensitive signals were considered positive for hTR expression.

**RESULTS**

Telomerase activity was detected in 14 (70%) of the 20 renal cell carcinomas tested. No correlation between telomerase activity and the histopathological parameters studied could be documented (Table 1). Of the paired normal renal tissue samples, all 20 samples were found to be telomerase negative.

We next examined the expression of the telomerase subunits hTR and hTERT in renal cell carcinoma and corresponding normal renal tissue samples. Using RT-PCR, expression of hTERT was detectable in all cancer and noncancer samples. Interestingly, although renal cell carcinomas U17T and U15T were found to be telomerase positive, U46T was telomerase negative.

**Table 1** Relation between telomerase activity (TA) status and histopathological stage and grade in the 20 renal cell carcinomas studied

<table>
<thead>
<tr>
<th>TA status</th>
<th>+</th>
<th>−</th>
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<tbody>
<tr>
<td>pT1/2</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>pT3/4</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>G1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>G3</td>
<td>3</td>
<td>3</td>
</tr>
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</table>

**Fig. 1** Representative results of RT-PCR analysis for the expression of the telomerase subunits hTR and hTERT in three renal cell carcinomas. Expression of GAPDH was used for control. Interestingly, although renal cell carcinomas U17T and U15T were found to be telomerase positive, U46T was telomerase negative.

**Table 2** Relation between telomerase activity (TA) status and expression of the telomerase subunits hTR and hTERT in normal and cancer renal tissue samples

<table>
<thead>
<tr>
<th>TA status</th>
<th>Normal renal tissue</th>
<th>Cancer renal tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>hTR+</td>
<td>hTR+</td>
</tr>
<tr>
<td>−</td>
<td>hTR−</td>
<td>hTR−</td>
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<tr>
<td>hTERT+</td>
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</tr>
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<tr>
<td>hTR−</td>
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<td>0</td>
</tr>
<tr>
<td>hTERT+</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>hTERT−</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

**Statistical Analysis.** Statistical analysis was performed using a χ2 test to evaluate significance between telomerase activity status and tumor stage or grade. P < 0.05 was considered statistically significant.
normal tissue, hTR was found to be exclusively expressed in the epithelial cells of the tubules but not in the glomeruli and in the cells of the Henle’s loop. Stromal cells and mature lymphocytes were also negative.

Using RT-PCR, expression of hTERT mRNA was demonstrated in 18 (90%) of the 20 cancer samples but also in 15 (75%) of the 20 noncancer samples. The association between hTERT mRNA expression and telomerase activity is given in Table 2. It is remarkable that 4 of the 18 hTERT mRNA-expressing cancer samples were found to be telomerase negative as were all normal samples also expressing hTERT. Of the two hydronephrotic kidneys, which were studied for control, telomerase activity was detected in none. One kidney showed hTR expression; none revealed expression of hTERT mRNA.

To study whether there is any relation between the hTERT mRNA expression levels and telomerase activity, we performed a semiquantitative RT-PCR for 30 cycles on eight cancer and corresponding normal tissue samples. The hTERT:GAPDH RT-PCR signal intensity ratios, normalized to those obtained with 100 ng of DU145 RNA (set to 100%), varied considerably from 0.08 to 0.80% in the cancer samples and from 0.08 to 6.1% in the normal samples (Fig. 3). Although the hTERT mRNA expression levels were approximately equal between the corresponding cancer and noncancer tissue samples, the strongly elevated levels in three normal samples (U47K, U56K, U96K), which exceeded the ones of the corresponding cancer samples, were striking, considering that they lacked telomerase activity.

**DISCUSSION**

In this study, we investigated the association between telomerase activity and the expression of hTR and hTERT.
mRNA in paired normal and neoplastic renal tissue samples. Our results on telomerase activity in renal cell carcinoma are in accordance with the data obtained by other groups (for literature see "Introduction"). Similar to our previous observations on 35 tumor nephrectomy specimens (20), telomerase activity in the current series was detected in 70% of the renal cell carcinoma samples. Thus, including our recently published series, the overall frequency of telomerase activity in renal cell carcinoma was 73%. No correlation between telomerase activity and clinicopathological characteristics of the tumors could be documented, with the histopathological staging and grading being fairly equally distributed in the telomerase-positive and -negative tumor groups. No telomerase activity was observed on TRAP assays of the paired normal kidney samples.

Using RT-PCR, expression of hTR was detected both in cancer and in normal renal tissue. This finding confirms most recent studies revealing hTR to be constitutively expressed in renal tissue, both normal and neoplastic (15, 34). Remarkably, using in situ hybridization, the expression levels of hTR were found to be markedly higher in the normal tissue than in the tumors. This finding is divergent to what was observed in other tissues (27, 33). hTR up-regulation thus does not seem to be necessarily related to telomerase activation, although organ-specific mechanisms of telomerase activation may exist. Concerning identity of hTR-expressing cells in the normal tissue, hTR expression was exclusively found in the epithelial cells of the tubules but not in those of the Henle’s loop and of the glomeruli.

Concerning the catalytic subunit of telomerase, we have demonstrated a high frequency of hTERT mRNA expression by RT-PCR in renal cell carcinoma (90%) and, notably, also in the normal parenchyma (75%) from cancer-bearing kidneys. The high frequency of hTERT mRNA expression particularly in the normal renal parenchyma is unexpectedly high and discordant to the detection rate of telomerase activity. Four of the 18 cancer samples with hTERT mRNA expression lacked telomerase activity in the TRAP assay (Table 2). Of the corresponding normal renal tissue samples, hTERT mRNA expression was detected in 15 samples, all of which were shown to be telomerase negative. When cancer and noncancer tissue samples were combined, a concordance rate of 52% was observed for hTERT expression and telomerase activity.

Our findings show that the expression of hTERT mRNA is not sufficient to produce an active telomerase enzyme, at least in renal tissue. This is in contrast to various studies reporting a strong correlation of hTERT mRNA expression with telomerase activity in neoplastic and nonneoplastic tissues of different origin. As an example, in hepatic tissue samples, including tumor and nontumor tissues, the levels of hTERT mRNA expression and telomerase activity showed good correlation, and there was no nontumor liver tissue that expressed hTERT (12). On the other hand, there are some reports pinpointing expression of hTERT mRNA in normal and cancer tissues without detectable telomerase activity. Kyo et al. (35) reported some cases of telomerase-negative ovarian cancer that revealed levels of hTERT mRNA expression equivalent to those in telomerase-positive cases. Concerning nontumor tissues, Ulaner et al. (36) most recently described hTERT mRNA in 16 of 20 normal ovarian tissue samples, despite the fact that all samples lacked telomerase activity. Similarly, Tahara et al. (37) described expression of hTERT mRNA and protein in normal telomerase-negative colorectal tissue. Interestingly, although there are data indicating a certain threshold level of hTERT mRNA that is required for telomerase activity (32, 38), Tahara and coworkers stressed the observation of equal hTERT expression levels in normal and tumor colorectal tissue, although telomerase activity was observed only in the tumor part but not in the normal.

This prompted us to perform semiquantitative RT-PCR to answer the question whether there is a relation between the hTERT mRNA expression levels and telomerase activity in renal tissue. Sufficient material of paired (tumor and normal) tissue samples was available from eight patients, with the study revealing no association between the amount of hTERT mRNA and detectable telomerase activity. Although the hTERT mRNA expression levels in the normal tissue samples were almost equal or even higher than those in the cancer samples, telomerase activity was only detected in the cancer samples but not in the normal ones. In addition, regarding the telomerase-positive cancer samples, it is worth noting that no association could be documented between telomerase activity and the amounts of hTERT mRNA.

Although no quantitative methods for telomerase activity were applied, our findings indicate that, similar to the hTR expression level, the level of hTERT mRNA expression does not play a critical role in determining telomerase activity in renal tissue. The lack of telomerase activity in normal renal tissue expressing hTR and hTERT mRNA suggests that other mechanisms, such as posttranscriptional modification of hTERT (9, 36, 39) or inactivation of inhibitors of the telomerase (40), are involved in the acquisition of enzymatic activity. This assumption is supported by the study of Kanaya et al. (15), who also observed hTERT expression in nontumor renal tissue samples despite their lacking telomerase activity. Although their frequency of 16% was lower than the one we observed, the biological relevance of hTERT mRNA expression in normal...
Telomerase Subunits in Renal Tissue

...renal tissue samples remains to be clarified. This question becomes particularly interesting in the three nephrectomy cases, where we observed an up to 20-fold increase of the relative hTERT mRNA expression level in the nontumor tissue samples compared with the tumor samples. Although each specimen tested was histologically controlled, we of course cannot fully exclude that because the extracts used for TRAP assay and RT-PCR were obtained from different portions in the same specimens, the discordances between telomerase activity and hTERT expression might, to some part, reflect heterogeneity of the cellular composition of the specimens. Another aspect of elevated hTERT expression in the normal renal tissue samples that should be considered is the presence of activated lymphocytes, which may express hTERT and consequently also telomerase activity. This, however, seems rather unlikely because we, like others (32), could not find a correlation between the degree of lymphocytic infiltrate and detectable telomerase activity or hTERT transcript. It is intriguing to speculate whether there may be an association between elevated hTERT expression in (histologically) normal renal cells and the risk of multifocal tumor occurrence.

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