Differential Expression of Full-length Telomerase Reverse Transcriptase mRNA and Telomerase Activity between Normal and Malignant Renal Tissues

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Abstract

Activation of telomerase, a key event during immortalization and malignant transformation, requires expression of the telomerase reverse transcriptase (hTERT). Consistently, lack of telomerase activity and hTERT expression occurs in most normal human somatic cells. However, it has been observed that both normal and cancerous renal tissues express hTERT whereas only the latter exhibits telomerase activity. The mechanism underlying the dissociation between hTERT expression and telomerase activity is unclear. In the present study, we examined telomerase activity and alternative splicing of hTERT transcripts in renal cell carcinoma (RCC) specimens and adjacent normal tissues from 33 patients with RCC. Telomerase activity was detectable in 27 of 33 (82%) RCC samples but none in their normal counterparts. Thirty-two of 33 tumors expressed overall hTERT mRNA and 27 of them contained full-length hTERT transcripts, all with telomerase activity. Although 42% (14 of 33) of normal renal samples expressed hTERT mRNA, none of them had full-length hTERT transcripts, coinciding with lack of telomerase activity. The presence of full-length hTERT mRNA and telomerase activity was significantly associated with c-MYC induction. In tumors, absence of full-length hTERT mRNA or telomerase activity defines a subgroup of nonmetastatic, early-stage RCCs. Taken together, telomerase repression in normal renal tissues is attributed to the absence of full-length hTERT transcripts, whereas telomerase activation is achieved via induction of or switch to expression of full-length hTERT mRNA during the oncogenic process of kidneys, and associated with aggressive RCCs.

Human linear chromosomes terminate with telomeric TTAGGG repeats and these repetitive DNA sequences progressively shorten with cellular divisions, by which limited life span or replicative capacities of normal human cells are brought about (1). During the oncogenic process, the maintenance of telomere length is required for transformed cells to override the intrinsic replicative barrier and to acquire immortal phenotype, and in vast majority of cases, this is achieved through activation of telomerase, a ribonucleoprotein DNA polymerase responsible for de novo synthesis of telomeric repeats TTAGGGS (2, 3).

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Telomerase is a multicomponent complex, and the catalytic holoenzyme consists of the telomerase RNA template and the catalytic subunit, telomerase reverse transcriptase (hTERT; ref. 4). hTERT has been shown to be a determinant for telomerase activity control, and the induction or introduction of hTERT expression is a prerequisite for acquisition of telomerase activity (5–7). Consistently, the absence and presence of hTERT expression have been intimately associated with telomerase repression in many types of normal human cells and activation in various malignant tissues, respectively (8).

Like other human tumors, most renal cell carcinomas (RCC) exhibit telomerase activation, thereby undergoing infinite proliferation, and a close correlation between hTERT expression and telomerase activity is well documented (9–17). It has been repeatedly observed, however, that up to 71% of normal kidney tissues taken from adjacent parts of RCC sites similarly contain hTERT mRNA without detectable telomerase activity. Information on qualitative difference(s) in hTERT expression between normal and malignant renal tissues and on mechanism(s) underlying the dissociation between hTERT expression and telomerase activity in normal renal tissues is still lacking. hTERT expression is predominantly governed at transcriptional levels, but potential roles for posttranscriptional modifications in the regulation of hTERT expression and telomerase activity have recently been established (18, 19). A number of studies have shown the presence of different transcripts of the hTERT gene in various kinds of human tumors and tissues.
further evidence has accumulated that only a full-length hTERT protein is catalytically active and therefore associated with telomerase activity, whereas all other spliced hTERT variants disrupt the reverse transcriptase domain and result in a truncated dysfunctional protein (22, 23). Alternative splicing of hTERT mRNA has been shown to contribute to the regulation of telomerase activity during embryonic development (21, 24) and in certain types of malignancies including ovarian carcinomas, neuroblastomas, and others (20, 25–27).

In the present study, we sought to elucidate whether alternative splicing of hTERT transcripts contributes to the differential expression of telomerase activity between normal and malignant renal tissues, and if so, whether a switch to full-length hTERT mRNA variant is associated with abnormal activation of the c-myc oncogene, a key transactivator for the hTERT gene. Additionally, we wanted to investigate potential relationships between telomerase activation or full-length hTERT mRNA induction and clinical/pathologic characteristics of RCCs.

### Materials and Methods

**Cell lines and cell culture.** Four tumor cell lines, A498, KRC/Y, CAKI-2, and TK10, all derived from RCCs, were maintained at 37°C in DMEM medium (Life Technologies, Paisley, United Kingdom) containing 10% FCS, 2 mmol/L L-glutamine, and antibiotics (100 units/mL

### Table 1. Patient and tumor characteristics, telomerase activity, overall and full-length hTERT mRNA expression in RCCs, and adjacent normal renal tissue

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (y)/Gender</th>
<th>Size (cm)</th>
<th>Stage</th>
<th>TA*</th>
<th>hTERT-All</th>
<th>hTERT-fl</th>
<th>Adjacent normal tissue</th>
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</table>

*TA, telomerase activity. The enzyme activity was assessed by using the telomerase-ELISA kit and its levels were arbitrarily expressed as absorbance ($A_{450} - A_{690}$). The absorbance values below 0.03 (less than 3-fold background/negative readings) were defined as negative for telomerase activity.

^hTERT-All, overall hTERT mRNA including all splicing forms of hTERT transcripts.

^hTERT-fl, full-length hTERT mRNA.

^xM, with metastases.

^k Not done.
penicillin and streptomycin). Cells at logarithmically growing phases were harvested for hTERT mRNA and telomerase activity analyses.

**Patients and tissue specimens.** The study included 33 patients with primary clear cell RCCs and was approved by the local ethics committee. All the patients were treated with radical nephrectomy at Qilu Hospital, Shandong University, between 2002 and 2003. After surgery, the tumor specimens and adjacent normal renal tissues were stored at −80°C until use. For each RCC, histology, tumor size (diameter), Robson stage, and regional and distant metastases at nephrectomy were evaluated. Patient and disease characteristics are detailed in Table 1.

**Telomerase activity assay.** A commercial Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) was employed to determine telomerase activity according to the protocol of the manufacturer. We used 0.5 µg of protein per assay and 28 PCR cycles for primary specimens, under which conditions the PCR amplification was maintained in a linear range and levels of telomerase activity could be semiquantified, based on our earlier studies (28). For analysis of telomerase activity in cell lines, the same amount of protein but 25 PCR cycles were carried out.

**RNA extraction, reverse transcription, and PCR.** Total cellular RNA in the tissue specimens was extracted using the ULTRASPEC-II RNA kit (Biotecx Lab., Houston, TX). cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden) and M-MLV reverse transcriptase. cDNA derived from normal adult kidneys was purchased from Clontech (Mountain View, CA) and PANOMICS (Redwood City, CA), respectively. The PCR primer sequences specific for all the variants of hTERT mRNA (accession no. AF015950) were 5′-CGG AAG AGT GTC TGG AGC AA (1,784-1,803, forward) and 5′-GGA TGA AGC GGA GTC (1,928-1,910, reverse; ref. 7; Fig. 1A). To amplify a full-length hTERT transcript, we used the following primer pair as described by Krams et al. (refs. 26, 27; Fig. 1A): 5′-TGT ACT TTG TCA AGG TGG ATG TG-3′ (2,172-2,194, forward) and 5′-GTA CCG CTT GAG GTC (2,371-2,350, reverse). PCR was done with the use of 36 and 37 amplification cycles for the primer pairs 1,784/1,928 and 2,172/2,371, respectively. The reverse transcription-PCR for the oncogene c-MYC mRNA was done as described (29), and the primer sequences were forward: TAC CCT CTC AAG AAC AGC AGC TGG CCC AAC AAT TCC T and reverse: TCT TGT CAT TCT CCT CGG TGT CGG AGG ACCT, which led to the amplification of a DNA fragment with 479 bp. A total of 30 PCR cycles at 95°C for 15 seconds, 60°C for 45 seconds, and 72°C for 60 seconds was carried out. β-Actin expression was used as a control for RNA loading and reverse transcription efficiency and amplified with its specific primers using 25 cycles. PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and visualized in UV light.

**Results.**

**Telomerase activation in renal cell carcinomas but not in normal adjacent renal tissues.** By using a telomerase ELISA kit, we measured telomerase activity in 33 RCC tumor samples and their corresponding normal tissues, and the obtained results are shown in Table 1. In 6 of 33 tumors, telomerase activity levels, arbitrarily expressed as absorbance (A450-A590) values, were just slightly higher (<0.02) than negative controls (<0.01), and they were thus defined as telomerase-negative RCCs. The remaining 27 tumor specimens (82%) exhibited various degrees of telomerase activity ranging from 0.07 to 0.56 (arbitrary absorbance values, Table 1). According to the same criteria, all 32 examined adjacent normal renal specimens contained no telomerase activity (Table 1), which is consistent with previous observations (11, 14–17).

We found no relationships between telomerase activity and age of patient or gender. However, tumor size was significantly correlated with telomerase activation (Table 2). Six of 12 RCCs (<5.0 cm (50%) were negative for telomerase activity whereas...
none of 21 tumors (with size) ≥5 cm lacked telomerase activity (P < 0.001, RCCs <5.0 versus ≥5.0 cm). Although there were no statistically significant differences in telomerase expression regarding stages and metastasis statuses, all six RCCs without telomerase activity belonged to stage I whereas all eight tumors at stages II to IV exhibited telomerase activity. Moreover, all six telomerase-negative tumors were among 29 RCCs without metastases whereas telomerase was activated in all four tumors with metastases (Table 2).

**Telomerase reverse transcriptase mRNA expression in both renal cell carcinomas and normal adjacent renal tissues.** Earlier clinical investigations have shown that telomerase activity is only detectable in RCC tumors but not in adjacent normal renal tissues, however, both normal and malignant tissues may express hTERT mRNA, a rate-limiting component for telomerase activity (11, 15, 17). To determine whether this was also the case for our cohort of patients, we employed the specific hTERT primers 1,784/1,928, used in above studies (15, 17), to PCR amplify hTERT transcripts in 33 RCC tumors and their corresponding normal tissues. In RCC specimens, 32 of them contained hTERT mRNA, as shown by the positive PCR amplification (Fig. 1B; Table 1). Twenty-seven of hTERT-expressing tumors concomitantly exhibited telomerase activity. Despite undetectable telomerase activity in all normal renal specimens, 42% of them (14 of 33) expressed various amounts of hTERT mRNA (Fig. 1B; Table 1). These results show a strong correlation between hTERT induction and telomerase activation in RCCs and at the same time a clear dissociation of two events in the corresponding normal specimens.

To further determine whether truly normal adult kidney tissues also express those hTERT transcripts, we did PCR on commercially available cDNA that was derived from healthy adult kidneys (free from diseases). The specific hTERT signal was seen in both cDNA samples (Fig. 1C).

**Lack of telomerase inhibitors in most normal telomerase reverse transcriptase-expressing renal tissues.** It is known that there may exist telomerase or PCR inhibitors in primary tissues, which likely interfere with the assessment of telomerase activity (8). In addition, a few types of tissues or cells contain too much RNase that may result in rapid degradation of various RNA including telomerase RNA templates (30). Under either circumstance, telomerase activity present in given specimens will frequently become undetectable. To determine whether such factors may explain the lack of telomerase activity in normal renal tissues, we mixed 0.5 μg protein extracts from each normal hTERT-expressing renal sample with the same amount of proteins derived from its paired RCC specimen, and then measured telomerase activity. As shown in Fig. 2, in 11 of 14 (80%) patients, the addition of normal renal protein extracts did not abolish telomerase activity in corresponding tumors. Telomerase activity was partially inhibited in only three tumors (patients 8, 14, and 24) in the presence of the protein extract derived from their adjacent normal renal specimens. Taken together, we conclude that the normal hTERT-expressing renal tissues indeed lack telomerase activity.

**Presence of full-length of telomerase reverse transcriptase transcripts in renal cell carcinomas but not in adjacent normal renal tissues.** Recent studies have revealed important roles for alternative splicing of hTERT mRNA in the regulation of telomerase activity (20–23, 26, 27). It has been established that there are at least five different variants of hTERT transcripts, among which only full-length mRNA encodes a functional hTERT protein essential for the active enzyme (22, 23). Furthermore, we realized that in previous reports only overall hTERT mRNA expression was analyzed, and no distinction was made between the full-length and other variants (11, 15, 17). With this in mind, we wanted to identify whether the expressed hTERT mRNA seen in both normal and malignant renal tissues, as detected by using reverse transcription-PCR with the primer pair 1,784/1,928, contained a full-length transcript. Because a positive PCR signal can be derived from the presence of any hTERT mRNA variants by using the primers 1,784/1,928 (Fig. 1A), we took another primer pair, 2,172/2,371 (Fig. 1A), unique to full-length hTERT mRNA (26, 27), to carry out PCR analyses by using the same batch of cDNA. Full-length hTERT

*TA, telomerase activity; hTERT-fl, full-length hTERT mRNA.
*†Fisher’s exact test using the SigmaStat program.
mRNA was detectable in 27 of 33 RCC samples, and all of them were hTERT-expressing tumors (Table 1; Fig. 1B). In sharp contrast, all adjacent normal renal specimens expressed no full-length hTERT transcripts regardless of the presence or absence of other hTERT mRNA variants (Table 1; Fig. 1B). Similarly, there was no full-length hTERT mRNA in cDNA samples derived from completely normal kidneys (Fig. 1C). Twenty-seven RCCs with full-length hTERT mRNA all had telomerase activity, whereas six tumors lacking full-length transcripts were negative for telomerase activity, indicative of a strong association between telomerase activation and expression of full-length hTERT mRNA. On the other hand, the transcriptional repression of full-length hTERT mRNA correlated with the absence of telomerase activity in normal renal tissues.

When full-length hTERT mRNA expression was related to the clinical/pathologic variables of the patients, it was found that its presence significantly correlated with tumor size (Table 2). There was a tendency for full-length hTERT mRNA expression to be associated with higher stages and metastatic RCCs, although not statistically significant (Table 2). The findings were completely consistent with those observed for telomerase activity in normal renal tissues.

Close relationship between c-MYC induction and full-length telomerase reverse transcriptase mRNA expression/telomerase activation in renal cell carcinomas. Because the oncogene c-MYC is a key player in the transactivation of the hTERT gene (18, 19), we further analyzed a potential relationship between c-MYC and full-length hTERT mRNA expression or telomerase activation. cDNAs from 18 RCC specimens and corresponding normal tissues were available for c-MYC mRNA analysis. None of the 18 normal renal samples expressed detectable c-MYC mRNA whereas 17 of 18 tumors exhibited abundant c-MYC transcripts (Fig. 3). All 17 c-MYC–expressing tumors were positive for full-length hTERT mRNA and telomerase activity, corroborating a striking correlation between c-MYC expression and presence of full-length hTERT transcripts/telomerase activity.

Concomitant expression of telomerase activity and full-length telomerase reverse transcriptase mRNA in renal cell carcinoma cell lines. To further define a relationship between telomerase activity and full-length hTERT mRNA expression, we did the same analysis on four renal cancer cell lines, A498, KRC/Y, CAKI-2 and TK10. All these RCC cell lines expressed telomerase activity (Fig. 4A). Moreover, both overall and full-length hTERT transcripts were readily detected in them by using the specific primer pairs (Fig 4B). Thus, the expression of full-length hTERT transcript is intimately associated with telomerase activation not only in primary RCCs but also in in vitro cultured RCC-derived cell lines.

**Discussion**

The stabilization of telomeres through activation of telomerase is the most common mechanism for cancer cells to escape the senescence checkpoint and to achieve replicative immortality. During the oncogenic process, the induction of expression of hTERT, the catalytic component, is required for acquisition of telomerase activity (3, 8). Consistently, a number of clinical investigations have shown that telomerase activation and hTERT expression occur in up to 90% of examined RCCs (9–17). However, one confusing observation in those studies was that normal renal tissues adjacent to the tumors lacked telomerase activity but frequently expressed hTERT mRNA (11, 17). Here we further corroborated the same scenario in our cohort of patients with RCCs: the coincidence of telomerase activity and hTERT expression was seen in most RCCs whereas 42% of normal adjacent renal tissues contained hTERT mRNA without detectable enzyme activity. A major objective of the present study was to elucidate this intriguing observation.
It has recently been revealed that alternative splicing of hTERT mRNA, a posttranscriptional mechanism, is actively involved in the regulation of telomerase activity in both normal and malignant human cells/tissues (20–23, 26, 27). At least five hTERT mRNA variants have been identified including the full-length (α + β+), α, β, α-β, and γ forms. The first four types of hTERT transcripts are found in various kinds of cells whereas the γ-form variant seems to be restricted to liver tissues (24). Interestingly, a full-length hTERT mRNA is the only transcript that is capable of being translated into a catalytically active protein. Because α, β, and α-β variants result in truncations or mutations in the reverse transcriptase domain essential for catalytic activity, they are either nonfunctional (β form and α-β form) or even have dominant-negative effects (α form; refs. 22, 23). During the embryonic development of human kidneys, hTERT-β mRNA can remain at a relatively high level after the disappearance of telomerase activity and full-length hTERT mRNA (21). Taken all these observations into consideration, we reasoned that hTERT mRNAs found in normal renal tissues are likely developmental residues of nonfunctional hTERT transcripts. Indeed, full-length hTERT mRNA was absent in all 33 examined normal renal specimens adjacent to tumor sites but prevalent in 27 of 33 hTERT-expressing RCCs, which highly correlated with the repression and activation of telomerase in normal and cancerous renal tissues, respectively.

Little is known about mechanisms and implications underlying active transcription of nonfunctional hTERT mRNA variants in normal renal tissues. RCCs may evolve from renal tissues with and without alternatively spliced hTERT mRNA, and under either circumstance, induction of or switch to full-length hTERT mRNA expression is required for telomerase activation during the oncogenic process. Regulatory pathways governing full-length hTERT expression are currently unclear. It seems that the frequency to acquire full-length hTERT mRNA expression in RCCs is unrelated to the presence of nonfunctional hTERT mRNA variants in their corresponding normal tissues, as shown in the present study. Rather, the induction of the oncogene c-MYC was closely associated with the expression of full-length hTERT transcripts. Given its important role in the transactivation of the hTERT gene, c-MYC may actively participate in the selective up-regulation of full-length hTERT mRNA expression in RCCs. Consistent with our finding, N-myc gene amplification has been shown to be highly related to telomerase activation and full-length hTERT mRNA expression in neuroblastosomas (26, 31). However, we indeed found that one RCC tumor (patient 13, Fig. 3) expressed full-length hTERT transcripts with no accompanying c-MYC mRNA induction, indicating that c-MYC is unlikely the sole driving force for the expression of full-length hTERT mRNA. A previous study has shown that transforming growth factor-β signaling pathway is frequently impaired in RCCs, it will be interesting to elucidate potential relationships of the defective transforming growth factor-β signaling pathway with telomerase activation during RCC development.

The frequent finding of alternatively spliced hTERT transcripts in various tumor types, as shown in previous studies (6, 20), indicates that a splicing control of hTERT mRNA for telomerase inactivation may occur in many human tissues in addition to the kidney. In normal colorectal tissues, for instance, abundant expression of hTERT mRNA was found but telomerase activity was undetectable (33). Because only overall hTERT expression was determined, it was unknown whether full-length hTERT transcripts were present in the examined normal specimens (33). According to our finding in normal renal specimens, it is likely that normal colorectal tissues contain no full-length hTERT mRNA, which is consistent with observed telomerase repression. Moreover, such a dissociation between telomerase and hTERT expression is sometimes even reported in cancer tissues when the overall and full-length hTERT mRNAs are not discriminated. This is apparently confusing and misleading. To avoid this, we thus suggest that the overall and full-length hTERT mRNA analyses should be simultaneously done in future clinical and other related studies.

Age, certain morphologic characteristics, tumor size, stage, nuclear grade, and metastasis status have been reported to be of prognostic significance in RCCs (34, 35). However, the clinical outcome of patients with RCC shows a broad variation, and more reliable predictors of prognosis are required. The present study shows a close correlation between telomerase activation or full-length hTERT transcript and tumors ≥5.0 cm, higher stages, and metastatic RCCs. Although the finding is not totally conclusive due to too few patients with advanced disease, the presence of telomerase activity and/or full-length hTERT mRNA apparently indicates progressive or aggressive RCCs. It has been shown that telomerase activity and full-length hTERT expression are powerful predictors in neuroblastosomas (26, 31) and certain types of malignancies (36–38). Therefore, it will be interesting to define whether these two variables will independently add prognostic information in larger cohorts of patients with RCC.

In summary, the present study shows, on the one hand, a striking correlation between full-length hTERT mRNA expression and telomerase activation in RCCs, and on the other hand, absence of full-length hTERT transcript despite the expression of alternatively spliced hTERT variants in normal renal tissues. Given the requirement of full-length hTERT for telomerase activity, our finding provides explanations for earlier confusing observations that normal renal samples expressed hTERT but lacked active telomerase. c-MYC induction is closely associated with the presence of full-length hTERT mRNA in RCCs, indicative of its potential roles in regulating hTERT mRNA splicing switch during development of RCCs. Finally, telomerase activation and/or full-length hTERT expression significantly correlated with bigger tumor size (≥5 cm) and advanced disease, which may improve our ability to better define patients with progressive RCCs.

References
Differential Expression of Full-length Telomerase Reverse Transcriptase mRNA and Telomerase Activity between Normal and Malignant Renal Tissues

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