Genetic Changes and Telomerase Activity in Human Renal Cell Carcinoma

Volker Rohde, Hans-Peter Sattler, Birgit Oehlenschläger, Stefan Forster, Thomas Zwergel, Gerhard Seitz, and Bernd Wullich

Clinic of Urology and Pediatric Urology [V. R., T. Z., B. W.] and Institute of Human Genetics [H-P. S., B. O., S. F.], University of the Saarland, 66421 Homburg/Saar, and Institute of Pathology, 96049 Bamberg [G. S.], Germany

ABSTRACT

Using the sensitive telomeric repeat amplification protocol assay, we detected telomerase activity in 26 of 35 (74.3%) renal cell carcinomas analyzed. Subdivision of the tumors according to telomerase activity did not reveal an obvious association between the presence of telomerase activity and histomorphological stage, grade, tumor size, or DNA ploidy. Furthermore, no association was found between telomerase activity and a distinct chromosomal aberration pattern; namely, loss of genetic material on the short arm of chromosome 3. Telomerase activity was also detected in 6 of 35 (17.1%) normal corresponding renal tissue samples, which seems interesting in light of the supposed biological role of telomerase expression in carcinogenesis. Interestingly, telomerase activity was detected in three of the four (75%) kidneys bearing non-clear cell tumor types, whereas the 31 kidneys with clear cell carcinomas, telomerase activity was found in only 3 (9.7%) normal tissue samples. In addition, the two renal angiomylipomas and one of the two analyzed transitional cell carcinomas of the renal pelvis were telomerase negative.

INTRODUCTION

Telomeres are specialized structures containing hexameric TTAGGG repeats at the ends of eukaryotic chromosomes. 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 about 50–100 bp with each cell division, because the 5' end of linear DNA cannot be fully replicated during S phase; this problem 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). At this time it is not exactly clear how shortened telomeres regulate the onset of senescence or programmed cell death.

In humans, germline cells compensate for the end replication problem by expressing the ribonucleoprotein telomerase (4). Telomerase is a reverse transcriptase containing a RNA template complementary to the TTAGGG repeat that allows telomeric sequences to be added to chromosome ends. In contrast to normal somatic cells, germline cells do not show substantial shortening of the telomeres with increased age. Telomerase is also detectable in many primary human tumor specimens and tumor-derived cell lines (for review, see Shay and Wright (5)). The expression of telomerase and ensuing stabilization of telomeres appear to be concomitant with the attainment of immortality, which is assumed to be critical in sustaining malignant tumor growth (6).

The recent development of the TRAP3 assay (7), a sensitive PCR-based method for detecting telomerase activity, has allowed for the screening of many different tumor types for telomerase activity. Summarizing the hitherto published data, telomerase activity was observed in 60–95% of malignant tumors (5). In addition, almost all immortalized cell lines analyzed thus far were shown to be telomerase positive; in contrast, mortal cell lines were found to be negative. Thus, telomerase activity appears to be reactivated in the process of immortalization and in most instances of tumorigenesis. Furthermore, at least in a subset of tumors, telomerase activity was found to be prognostically relevant, being correlated with poor clinical outcome (8–10).

The renal cell carcinoma is well known for its unpredictable behavior. This tumor shows different growth patterns, from early metastatic presentation to considerable local growth without invasion or distant manifestations for many years (11). In addition to the histopathological stage, which has been recognized as the single most prognostic factor, other parameters were evaluated in regard to their impact on renal tumor growth. Only cellular DNA content seems to be of predictive importance; aneuploidy was found to be associated with high proliferation, local tumor invasion, and poor prognosis (12). Furthermore, distinct chromosomal changes were also shown to be related to tumor progression in renal cell carcinoma (13).

In the present study, we examined telomerase activity, DNA ploidy, and chromosomal pattern in a series of 35 renal cell carcinomas. This comprehensive approach was chosen to answer the question of whether distinct chromosomal changes, including loss of genetic material on the short arm of chromo-
some 3, are related to telomerase activation in renal cell carcinoma. The study was motivated by in vitro transfection experiments that have shown that repression of telomerase function in chromosome 3p-deficient renal carcinoma cells could be achieved by reintroduction of a normal chromosome 3 (14).

MATERIALS AND METHODS

Tissue Samples. Tumor specimens were obtained from 35 patients who underwent nephrectomy for renal cell carcinoma. In four other patients, nephrectomy was performed for renal angiomyolipoma in two cases and for transitional cell carcinoma of the renal pelvis in the other two. For comparison, normal renal cortex samples were taken in each case. All specimens were obtained immediately after kidney removal in the operation theater. Tumor and renal cortex samples were divided into four parts: one part was snap frozen in liquid nitrogen and stored at −80°C until the TRAP assay was performed, one was used for flow cytometric DNA analysis, one was used for short-term cell culturing and consecutive cytogenetic analysis, and one was used for precise histopathological examination. Histopathological classification was done according to the TNM classification system (15). Preoperative examination of the patients included pulmonary X-ray, ultrasound, and computerized tomography revealing no distant metastasis. None of the patients had been treated with radiation, chemotherapy, or immunotherapy before nephrectomy. For control, five tissue samples of hydronephrotic kidneys without malignancy were assayed for telomerase activity.

Preparation of the Tissue Extract. Tissue extracts were obtained according to the procedure given by Piatyszek et al. (16) with minor modifications. Frozen tissue samples of 50 mg were homogenized in 200 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 12 mM EGTA, 0.1 mM 4(2-amino)benzenesulfonyl fluoride hydrochlorine, 0.5% CHAPS (Sigma Chemical Co., St. Louis, MO), and 10% glycerol). After 30 min of incubation on ice, the lysates were centrifuged at 16,000 x g for 30 min at 4°C. The supernatant was rapidly frozen and stored at −80°C. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL).

TRAP Assay. The TRAP assays were performed as described by Kim et al. (7). Briefly, 2 μl of the tissue extract (6 μg protein equivalent) were added to 46.5 μl of reaction mixture containing 50 μM dNTPs, 0.5 μM T4gene32 protein (Boehringer Mannheim, Mannheim, Germany), 5 μl of the TRAP assay buffer (20 mM Tris-HCl, 1.5 mM MgCl2, 62 mM KCl, 0.005% Tween 20, 1 mM EGTA), 0.1 mg/ml BSA, and 0.1 μg of TS primer. The oligonucleotides used (TS and CX primer) were described previously (16). The reaction solution was layered with mineral oil and incubated for 30 min at room temperature mediating extension of the TS primer. Afterward, they were heated to 90°C, and 0.1 μg of the TS primer and 2.5 units of Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) were added to each tube. Thirty-five PCR cycles were run under the following conditions: 94°C for 30 s, 50°C for 20 s, and 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.

The sensitivity of our nonradioactive TRAP assay was determined using serially diluted extracts from PC3 cells (Fig. 1). About 100 cells are sufficient for the detection of telomerase activity. PC3 is an established human prostatic carcinoma cell line.

As a positive control, 2 μl of a cell extract from the established human prostatic carcinoma cell line PC-3 with known telomerase activity were used. As a negative control, 2 μl of the lysis buffer without any cell extracts were assayed in

![Fig. 1 Sensitivity of nonradioactive TRAP assay. The TRAP assay was performed on serially diluted extracts from PC3 cells.](image)

**Table 1** Telomerase activity in 35 renal cell carcinomas given in relation to clinical and histopathological data

<table>
<thead>
<tr>
<th>Telomerase activity</th>
<th>Positive (n = 26)</th>
<th>Negative (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (yr)</td>
<td>40–85</td>
<td>47–76</td>
</tr>
<tr>
<td>Mean age</td>
<td>64.8</td>
<td>58.5</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>Chromophobic</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chromophilic</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>3–10</td>
<td>3–11</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>pT2</td>
<td>18</td>
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<tr>
<td>pT3</td>
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<td>2</td>
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<td>1</td>
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<tr>
<td>I</td>
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<tr>
<td>III</td>
<td>8</td>
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</table>
Fig. 2 Polycrylamide gel showing TRAP assays from extracts of renal tissue samples. Brackets enclose adjacent samples taken from patients who underwent nephrectomy for renal cell carcinoma (cases 1–4) and for hydronephrotic kidney without malignancy (case 5). T, tumor; N, normal corresponding renal tissue sample; H, hydronephrotic kidney without malignancy, with (+) or without (−) RNase pretreatment of the extract. Pretreatment with or without RNase A was used to confirm the specificity of the TRAP assay reaction. The renal cell carcinomas of cases 1–4 showed telomerase activity. No telomerase activity was detected in the hydronephrotic kidney. Note telomerase activity in the normal corresponding renal tissue samples of cases 2 and 3.

Table 2 Telomerase activity in relation to DNA ploidy

<table>
<thead>
<tr>
<th>Case</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>RNase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Each experiment. All extracts showing 6-bp ladders were tested for sensitivity to RNase pretreatment. 5 μl of a positive sample were incubated with 1 μl of RNase A (1 mg/ml) for 30 min at 37°C. Only samples that produced RNase-sensitive ladders extending 4 or more units were considered positive for telomerase activity (17). Confirmation of positive TRAP assay signals was demonstrated by repeat experiments.

All telomerase-negative samples were checked by another TRAP round including a 150-bp internal DNA standard (18) to exclude the presence of Taq polymerase inhibitors in the tissue extracts. To assess the possibility of tissue degradation as a further cause of negative telomerase activity, the alkaline phosphatase activity of all samples was analyzed according to the manufacturer’s instruction (Sigma). The assays were done for 30 μg of protein extract or CHAPS lysis buffer as a reference sample (16).

Flow Cytometric DNA Analysis. The detailed protocols were reported previously (19). Briefly, nuclear suspensions were isolated from frozen tissue samples by mechanical disaggregation. The fluorescence of the 4',6-diamidino-2-phenylindole dihydrochloride-stained nuclei was measured in a PAS III flow cytometer (Partec AG, Münster, Germany) and displayed as histograms of DNA content versus number of nuclei. As standard cells, we used residual diploid cells, which were still present in all analyzed samples. DNA ploidy was defined by one or more additional peaks, distinguished from the G0/G1 and G2/M peaks. The ratio of the mean channel number of the aneuploid cell population to the diploid cell population was calculated as the DNA index.

Short-Term Cell Culturing and Cytogenetic Analysis. Tumor and normal renal tissue samples were disaggregated mechanically and/or enzymatically. Cell culture was performed using Dulbecco’s modified Eagle’s MEM with 10% FCS. Mitotic cells were harvested after 4–18 days. Chromosome preparation and GTG banding followed standard protocols. The karyotypes were described according to the International System for Human Cytogenetic Nomenclature guidelines for cancer cytogenetics (20).

Statistical Analysis. Statistical analysis was performed using a χ² test to evaluate the significance of the differences. P < 0.05 was considered statistically significant.

RESULTS

Telomerase activity was detected in 26 of 35 (74.3%) renal cell carcinomas analyzed (Table 1). The telomerase-positive tissue extracts produced a characteristic 6-bp ladder as shown in Fig. 2. The two renal angiomyolipomas revealed no telomerase activity. Of the two transitional cell carcinomas of the renal pelvis, telomerase activity was detected in one.

In 29 (82.9%) of the 35 corresponding normal tissue samples that were derived from renal cell carcinoma-bearing kidneys, no telomerase activity could be detected. Six samples (17.1%), however, were positive; they originated from three kidneys with clear cell carcinomas, two with chromophilic carcinomas, and one with a chromophobe carcinoma. The four normal tissue samples from the kidneys with angiomyolipomas or transitional cell carcinoma of the renal pelvis were found to...
be telomerase negative, as were the five hydronephrotic kidneys without malignancy.

Extracts from all telomerase-negative tissue samples revealed a positive signal for the internal telomerase assay standard, excluding an inhibitory effect on the Taq polymerase. Tissue degradation as a further cause of negative telomerase activity was also excluded; all telomerase-positive and telomerase-negative renal tissue samples revealed alkaline phosphatase activity. Although varying levels of activity were seen, the difference in the levels between telomerase-positive and telomerase-negative samples was not statistically significant.

Subdivision of the tumors according to telomerase activity did not reveal any obvious difference in distribution of the histopathological parameters studied (Table 1). Histopathological staging and grading, as well as tumor size, were fairly equally distributed in both groups.

Applying DNA flow cytometry, 18 of 35 (51.4%) renal cell carcinomas were found to be diploid. An aneuploid DNA content was detected in 13 (37.1%) renal cell carcinomas and tetraploid in 2. Two renal cell carcinomas were not analyzable. Aneuploidy and telomerase activity could not be statistically correlated (Table 2).

In addition to the TRAP assay, cytogenetic analyses were performed on 11 renal cell carcinomas. Chromosomes of good banding quality were achieved in nine tumors, allowing for detailed karyotyping. No karyotyping was possible in two cases due to insufficient number of metaphases and to poor banding quality. The cytogenetic results are given in Table 3. Clonal structural and/or numerical chromosomal abnormalities were detected in all nine tumors. Aberrations of chromosome 3 resulting in loss of genetic material on the short arm were detected in five tumors (Fig. 3). No chromosome 3 aberration was found in three tumors: one case had a trisomy 7 as the sole aberration, one showed trisomy 7 in addition to other numerical chromosomal abnormalities.
somal aberrations (Fig. 4), and one revealed cytogenetically unrelated cell clones in addition to cells with trisomy 7. One tumor exhibited a trisomy 3 plus additional chromosomal changes. In the 11 normal renal cortex samples that were analyzed for comparison, a normal male/female karyotype was found. No association between telomerase activity and a distinct chromosomal aberration pattern was found.

**DISCUSSION**

In the present study, 35 renal cell carcinomas, 2 renal angio-myolipomas, and 2 transitional carcinomas of the renal pelvis were examined for telomerase activity by applying the TRAP assay. Until now, only one study on renal cell carcinomas has been published revealing telomerase activation in 71% of the tumors analyzed (21). This high frequency of telomerase activity in renal cell carcinomas is confirmed by our own findings. Telomerase activity was detected in 74% of the renal cell carcinomas analyzed, indicating that this enzyme plays an important role in the process of renal cell carcinoma development.

Subdivision of the tumors according to telomerase activity did not reveal an obvious association between the presence of telomerase activity and the histopathological parameters studied. Histopathological stage and grade, as well as tumor size, were distributed similar in the two groups. This finding is in accordance with the recently published data on renal cell carcinomas, but it differs from the studies on neuroblastomas and gastric and breast cancers (8–10). For these three tumor types, less advanced stages were found for the telomerase-negative tumors. Similar findings on prostatic carcinomas were reported by Sommerfeld et al. (17), who observed a strictly organ-confined growth of the telomerase-negative tumors.

On the basis of light and electron microscopic, immunocytochemical, and cytogenetic studies, several types of renal cell carcinomas can be differentiated that are thought to arise from different cell types of the nephron-collecting duct system (22). Of the 31 clear cell carcinomas analyzed, 23 (74.2%) were found to exhibit telomerase activity. Of the three chromophilic tumors, two were telomerase positive, as was the one chromophobic tumor studied. Interestingly, when the telomerase activity in histopathologically normal corresponding renal tissue samples was observed, telomerase activity was detected in 3 of the 4 (75%) kidneys bearing non-clear cell tumor types, whereas of the 31 kidneys with clear cell carcinomas, telomerase activity was found in only 3 (9.7%) normal tissue samples. Although the number of cases analyzed was too low to establish any statistically significant difference, it might be interesting to clarify whether telomerase activity in corresponding normal renal tissue may characterize a subset of renal cell carcinoma patients who are at high risk for a multifocal tumor occurrence. It is well known that chromophilic tumors tend to occur multifocally. We recently reported a case with bilateral and multifocal chromophilic renal tumors in which chromosome instability was detected in adjacent normal kidney tissue samples (23). This may indeed indicate genetic changes to occur in normal cells as a predisposing step in the pathogenesis of multiple renal tumors. On the other hand, there is increasing evidence that telomerase activity is not necessarily related to tumoral cell growth but can also reflect physiological hyperproliferation of normal somatic cells (24, 25). Thus, the biological relevance of telomerase-positive normal renal cells remains to be clarified. We are aware that the mere presence of tumor cells in our telomerase-positive normal tissue samples cannot be ruled out completely, but it seems rather unlikely. Thorough histopathological examination was performed on all specimens. Furthermore, DNA flow cytometry and metaphase cytogenetics revealed a normal chromosomal complement in all corresponding normal renal tissue samples.

When the DNA content was assessed by DNA flow cytometry, diploidy was found in 51.4% of the renal cell carcinomas and aneuploidy in 37.1%. Tetraploidy was detected in two
tumors. These findings are similar to previous studies (26). When DNA flow cytometric and metaphase cytogenetic results are compared, it is remarkable that the aneuploidy rate is higher when assessed by cytogenetics than by DNA flow cytometry (Table 3). In all DNA flow cytometry aneuploid renal cell carcinomas, an aberrant chromosomal pattern could be confirmed by cytogenetic analysis. Three DNA flow cytometry diploid tumors, however, revealed numerical and/or structural clonal chromosomal aberrations, indicating a higher sensitivity of metaphase cytogenetics for detecting aneuploidy, particularly in the near-diploid range, than DNA flow cytometry. A correlation between telomerase activity and DNA content or a distinct chromosomal aberration pattern was not found. It is especially noteworthy that no difference in telomerase activity could be discovered between renal cell carcinomas with and without cytogenetic loss of chromosome 3p material. Although alleloypning may allow researchers to identify more renal cell carcinomas with submicroscopic loss of loci on chromosome 3p, no principally different finding in regard to the relation of chromosome 3p alterations and telomerase activity is to be expected by this method.

No data about telomerase activity in renal angiomylipomas and transitional cell carcinomas of the renal pelvis have been published thus far. We found the two angiomylipomas to be telomerase negative. Telomerase activity, however, was detected in one of the two transitional cell carcinomas of the renal pelvis.

In summary, our results provide further evidence for the assumption that telomerase activity plays an important role in renal cell carcinoma growth. No correlation to DNA content and cytogenetic subtypes, particularly loss of genetic material on chromosome 3p, could be documented. However, experimental data indicate a by far more complex picture of cell senescence versus continued proliferation as proposed in the telomeric hypothesis. Telomerase activity alone does not maintain telomere length, and telomerase-independent mechanisms to stabilize chromosome ends must exist. Therefore, further studies on the biological role of telomerase activity in human cancer should include the investigation of possible other mechanisms of uncontrolled cell division.

REFERENCES

Genetic changes and telomerase activity in human renal cell carcinoma.

V Rohde, H P Sattler, B Oehlenschläger, et al.