Telomere Shortening in Renal Cell Carcinoma

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ABSTRACT

The ends of human chromosomes consist of a specialized structure, the telomere, composed of repeats of TTAGGG making up a total of 5–15 kilobase pairs, depending on age and proliferative activity of the tissue. The major function of telomeres is to provide stability to chromosomes and protect underlying unique coding sequences from degradation. There is a loss of telomeric sequences following every cell division estimated to be between 50 and 65 basepairs/cell division in human fibroblasts and embryonic kidney cells in vitro. This loss is due to the fact that DNA replication is incomplete for one strand at each telomere end. In lower eukaryotes there is a compensation mechanism provided by the enzyme telomerase, which is inactive in human somatic cells. Telomerase activation has also been detected in in vitro immortalized human cells.

In this study we analyzed renal cell carcinoma for the occurrence of telomere shortening using the probe (TTAGGG)4. Southern blots of HindIII-digested DNA revealed a shortening of mean telomere restriction fragment (TRF) length of 0.4 to 2.5 kilobase pairs in 2 or 3 intratumoral samples in all 10 tumors analyzed. No obvious intratumoral heterogeneity was found in mean TRF length values. However, heterogeneity was shown by the occurrence of at least two separate peak TRF values in 7 of 10 tumors, indicating the presence of different tumor cell clones. A conflicting observation was made when we evaluated the intensity of the hybridization signals, where three of the tumors showed an increase in hybridization signals despite concomitant TRF reduction. We found no correlation between tumor size and calculated tumor cell division numbers. In two tumors, the calculated cell division cycles were unrealistically low compared to the tumor size. These data suggest that telomerase activation might occur in human renal cell carcinoma.

INTRODUCTION

The structure of the chromosome ends has been highly conserved throughout evolution from unicellular eukaryotes to mammals. The telomeric DNA sequences consist of simple tandemly repeated units throughout evolution from unicellular eukaryotes to mammals. The integrity of telomeres is important for the completion of subtelomeric DNA. Cytogenetic studies have shown that cells with broken chromosome ends were unstable and prone to fuse end to end, leading to ring forms or dicentric chromosomes (5). The length of telomeres gradually decreases with increasing age and cell division number, which can lead to chromosome instability and genetic changes of possible significance for tumor development (5–7).

Futhermore, a marked reduction of telomere length has been reported in a subset of tumors as colorectal carcinoma, Wilms' tumor, and childhood leukemia (2, 7, 8), which can be a consequence of the increased number of cell divisions in these malignancies. Since lower eukaryotes like tetrahymena do not have any obvious shortening of the telomeres an enzyme activity with telomere lengthening properties was proposed. This enzyme, telomerase, was shown to be a ribonucleaseprotein for which the RNA component functions as a template for the synthesis of the telomere sequence in a reversed transcriptase manner (9). In humans the telomeres in germline cells are maintained at the same length during the lifetime of the individual, in contrast to the telomeres of somatic tissues (10, 11). It was proposed that the telomerase is inactive in somatic cells and that telomere shortening is related to the number of cell divisions a specific tissue has gone through (10).

A broad range of restriction enzymes are unable to cut within the distal TTAGGG telomeric repeats, since they seem to lack restriction enzyme cutting sites (2). The corresponding TRFs therefore consist of TTAGGG repeats and subtelomeric DNA with other nucleotide compositions. The subtelomeric DNA possesses moderate repetitive structures and has been estimated to be between 2.5 and 4 kilobase pairs long (2, 12).

We have previously shown that intratumoral heterogeneity is a common finding in renal cell carcinoma (13, 14). Because a reduction in telomere length can reflect the number of cell divisions in a cell population, we have hypothesized that it might be possible to find various subpopulations when analyzing TRF length, based on the assumption that there are cell populations with different growth properties.

In the present study we have been able to demonstrate a reduction in TRF length and heterogeneity of the length and volume of the telomeric DNA in renal cell carcinoma. There are also findings that indicate the possibility of activation of telomerase in a subset of tumors.

MATERIALS AND METHODS

Samples. Tumor DNA was isolated from two (four tumors) or three (six tumors) samples from different parts of sporadic renal cell carcinomas from 10 patients. Constitutional DNA was in each case isolated from normal renal cortex tissue from the same kidney. A part from each sample was used for histopathological examination.

DNA Isolation and Southern Transfer. High-molecular-weight DNA was prepared by digestion with protease K and extraction with phenol/chloroform. Equivalent amounts of tumor DNA and constitutional DNA (10 μg) were digested overnight with 5 units/μg of HindIII and HaeIII or AluI under conditions recommended by the manufacturers (Boehringer-Mannheim, Mannheim, Germany, and New England Biolabs, Beverly, MA). DNA fragments were separated by electrophoresis through 25-cm-long 0.7% agarose gels in 45 mM TBE buffer. The separated DNA fragments were depurinated in 0.25 M HCl for 15 min, denatured in alkali, and transferred to nylon membranes (Hybond-N; Amersham, Buckinghamshire, England) using 20× SSC (1 × SSC: 0.15 M NaCl, 0.02 M Na3Citrate). DNA denaturation and transfer to membranes was confirmed by UV illumination and X-ray film autoradiography. DNA was UV crosslinked to the membrane and baked at +80°C for 20 min. Membranes were prehybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, and 0.1% sodium dodecyl sulfate. Hybridization. Oligonucleotide probe (TTAGGG)4 (Symbicom AB, Umeå, Sweden) was 5'-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (USB, Cleveland, OH). Prehybridization and hybridization were performed at +48°C in 5× SSC, 5× Denhardt's solution, and 0.1% sodium dodecyl sulfate. The probe was added to the hybridization mixture and hybridized to the membrane at +48°C for 20 min. The membrane was washed twice for 30 min at room temperature in 2× SSC, 0.1% sodium dodecyl sulfate. After washing, the membrane was subjected to autoradiography to detect the specific hybridization signal.
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Fig. 1. Plotting of migration distance versus size in kilobase pairs of a DNA marker in a TRF gel with the corresponding formula. Abbreviations as described in “Materials and Methods.”

\[ D_m = 1283 + 6152 \times e^{-0.281 \times L_i} \]

Densitometry and Evaluation of the Length and Volume of Telomere Fragments. Autoradiographs were scanned with a densitometer (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA). Mean TRF length was defined as

\[ \sum \frac{OD_i}{\sum (OD_i/L_i)} \]

where \( OD_i \) is the densitometer output and \( L_i \) is the length of DNA in position \( i \). Sums were calculated over the range of 2–17 kilobase pairs. The size of the DNA fragments along each lane was calculated in Excel (Microsoft, Redmond, WA) using the formula

\[ D_m = c_1 + c_2 \times e^{-c_3 \times L_i} \]

proposed after examination of the migration pattern of size standards in MathLab (Mathworks, Natick, MA); where \( D_m \) is the distance migrated analyzed by Image Quant (Molecular Dynamics); \( c_1, c_2, \) and \( c_3 \) are constants 1, 2, and 3, respectively; and \( L_i \) is the size of the DNA fragments in kilobase pairs (Fig. 1). The constants were calculated for each autoradiogram using dodecyl sulfate using a thermostat-controlled hybridization incubator (Techno, Cambridge, England). Washes were performed in 4× SSC at room temperature and 4× SSC at 48°C. Filters were autoradiographed (Hyperfilm-MP; Amersham) with an intensifying screen at −70°C for 12–24 h.

Fig. 2. Analysis of TRF length in tumor 11/89. A, (CAC)\textsubscript{5} banding pattern. B, detection of telomere restriction fragments with (TTAGGG)\textsubscript{4}. C, corresponding TRF profile after compensation for loading differences using (CAC)\textsubscript{5} banding pattern as loading reference. N, constitutional DNA; T1, tumor sample 1; T2, tumor sample 2; T3, tumor sample 3; OD, optical density (absorbance).
Systat (Systat, Evanston, IL) before using the formula in Excel (Microsoft). Based on these calculations the size of the DNA at every position i can be determined. Telomere peak values were measured by estimating the band size corresponding to the point with the highest absorbance within the peak. Difference in TRF peak value was defined as a divergence of at least 1 kilobase pair between normal and the tumor sample in each peak.

By using the integration facilities in Image Quant (Molecular Dynamics), we were able to calculate the volume of the hybridized TTAGGG sequences.

In order to check for differences in DNA loading all filters were hybridized with a microsatellite probe (CAC)\textsubscript{5}. In case of differences, the tumor samples were normalized using the calculated compensation factor.

**Calculation of the Number of Cell Divisions.** Evaluation of possible tumor cell divisions undergone, based on difference in mean TRF length between normal and tumor samples, was made using the formula

\[
\text{TRF (kilobase pairs)} = 0.05 \times (\text{kb/division}) \times n \text{ (divisions)}
\]

modified after Levy et al. (15).

**RESULTS**

All tumor DNA samples were digested with two different restriction enzymes, giving only minor differences in estimated TRF lengths between the digests. None of the digested samples exhibited degradation of the DNA when hybridizing with the (CAC)\textsubscript{5} microsatellite probe to control for variation in loading and completion of digestion. Fig. 2 shows the hybridization of the (TTAGGG)\textsubscript{4} probe to Hinf1-digested DNA from matched normal kidney DNA and tumor samples, together with corresponding TRF profile and (CAC)\textsubscript{5} banding patterns.

In 5 of the 10 tumors two intensity peaks were observed within individual samples as detailed below and in Fig. 3. When calculating the mean TRF in these cases both peaks were included, since none of them seemed to correspond to the normal DNA peak, although they were separately noted when we estimated peak values. All tumors showed a reduction in the TRF length in all 25 intratumoral samples analyzed, ranging from 0.4 to 2.6 kilobase pairs (Table 1 and Fig. 4).

When measuring the mean TRF length there was no significant variation between samples from different regions in 9 of 10 tumors. One of the tumors (18/89) was aberrant, showing a deviation from the
normal sample of 1.2, 1.1, and 0.4 kilobase pairs, respectively, in the three tumor samples (Figs. 4 and 5).

Analysis of peak TRF values of the tumors revealed a more complex pattern compared with mean TRF values, since it demonstrated the heterogeneous nature of the tumors. Peak TRF value of all tumor samples deviated from the corresponding normal tissue (Fig. 3). In 7 of 10 tumors there were at least two subpopulations present, in five of which two different TRF peak values could be distinguished within individual samples. Thus, in 9 of the 25 tumor samples an intrasample heterogeneity was found. One example is tumor 13/89, showing two different TRF peak values in samples T2 and T3. These tumor samples were distinctly separated from the normal sample with 4 kilobase pairs or more (Figs. 4 and 5). A similar finding was observed in tumor 2/88. Three tumors (26/87, 18/89, and 2/89) also possessed two different subpopulations, where the difference between normal and tumor samples only was around 1–2 kilobase pairs for peak 1, whereas peak 2 showed a more pronounced deviation from the normal peak value. Furthermore, the T3 sample peak in tumor 18/89 did not deviate significantly from the normal peak. Thus, a considerable intratumoral heterogeneity in TRF peak values existed in 70% of the renal cell carcinomas.

When an estimated length of the subtelomeric sequences of 2.5 kilobase pairs (12) was subtracted from the mean TRF lengths, 6 of 10 tumors had between 2.5 and 3.0 kilobase pairs of “pure” telomeric TTAGGG sequences. Four of 10 tumors had less than 2.5 kilobase pairs, where tumor 23/87 was exceptional, with only 1.1 kilobase pairs of TTAGGG repeats.

In simian virus 40-transformed human fibroblasts and embryonic kidney cells a loss of 50 to 65 base pairs/cell division has been found (12, 15). Assuming a similar loss of 50 base pairs/cell division in renal cell carcinoma it was possible to calculate the approximate difference in number of cell divisions for tumor samples compared with normal cortex tissue, giving between 14 and 50 cell division rounds (Table 2). This means that, e.g., tumor 20/87, which according to the calculations had undergone only 14 cell divisions at the most, could contain $2^{14} \approx 16,000$ cells, although the tumor measured 150 mm in diameter. A similar finding in contradiction to the size of the tumor was observed for tumor 11/89. In contrast, the numbers of estimated cell divisions in other tumors were rather high compared with tumor size: e.g., tumor 26/87 with a size of 35 mm and a cell division number of 36, giving $6.9 \times 10^{10}$ cells. Thus, no correlation was found between the relative size of the tumors and the theoretical number of cell divisions the tumors had undergone.

After correction for DNA loading using the (CAC)5 control probe most tumor samples were aberrant in their total signal intensity compared with the normal samples. In 7 of 10 tumors the signals from all samples were significantly diminished (Fig. 6). In tumor 22/87 one sample was close to 100%, and the second sample signal was slightly increased, whereas the third sample gave a clearly reduced signal. Tumor 2/89 showed one sample with signal intensity comparable to the control and a second sample with significantly increased intensity. All three samples from tumor 11/89 gave increased signals (Fig. 2).
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A

B

C

Fig. 5. TRF-profiles obtained from autoradiograms in Fig. 4. A: tumor 23/87. B: tumor 13/89. C: tumor 18/89. Arrows, TRF-peak 1; filled arrowheads, TRF-peak 2. Abbreviations as in Fig. 2.

DISCUSSION

In the present study new and interesting information on TRF reduction and TRF peak values was obtained from renal cell carcinomas. From the analysis of the mean TRF length we can conclude that all tumors investigated had a decrease in TRF length in all of the intratumoral samples examined, with the exception of the T3 sample from tumor 18/89 (Table 1 and Fig. 4). Within individual tumors mean TRF values were similar for the different samples, indicating an overall similar mixture of cells (i.e., chromosomes) with various telomere lengths. Comparable TRF reductions have been reported for colorectal carcinoma and Wilms' tumor in studies of one sample from each tumor and normal tissue from the same individual (2, 7, 8).

Normal somatic cells have a replicative limit ("the Hayflick limit") in vitro, which is related to the age of the donor (reviewed in Ref. 5). In fibroblasts this limit is reached after about 50-80 doublings. One possible explanation for this phenomenon is that the telomeres become progressively shortened after each cell division. At a certain telomere length there is a checkpoint when the final cell cycle exit is signaled and the cells stop dividing. During the development of immortal cells this checkpoint is bypassed and the cells continue to divide until the telomeres are extremely short or even absent on some chromosomes, leading to a rise in chromosomal abnormalities and high cell death (5, 10, 12). In simian virus 40-transformed embryonic kidney cells an activation of the enzyme telomerase has been detected at this stage in surviving cells being immortalized (12). In HeLa cells and other permanent cell lines active telomerase also has been detected (Ref. 16 and the authors' unpublished data).

A similar scenario has been proposed for the progression of tumors in vivo, with oncogene activation and/or tumor suppressor gene inactivation inducing disturbed cell growth control and a possibility of bypassing the Hayflick limit. It is not known whether telomerase activation occurs in vivo in tumor cells.

Assuming that renal carcinoma cells in vivo, as fibroblasts and embryonic kidney cells in vitro, loose around 50 base pairs/cell division (12, 15), it might be possible to make a rough estimate of the number of cell divisions a tumor has passed through, as summarized in Table 2. For some of the tumors these calculations gave surprising

Table 2. Theoretical number of cell divisions and total cell numbers in the tumors (i.e., difference between normal sample and T1 sample for each tumor).

<table>
<thead>
<tr>
<th>Case</th>
<th>Size</th>
<th>ΔT1</th>
<th>No. of cell divisions</th>
<th>No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>20/87</td>
<td>150</td>
<td>0.7</td>
<td>14</td>
<td>1.6 × 10^6</td>
</tr>
<tr>
<td>22/87</td>
<td>40</td>
<td>1.2</td>
<td>24</td>
<td>1.7 × 10^7</td>
</tr>
<tr>
<td>23/87</td>
<td>110</td>
<td>2.5</td>
<td>50</td>
<td>1.1 × 10^15</td>
</tr>
<tr>
<td>26/87</td>
<td>35</td>
<td>1.8</td>
<td>36</td>
<td>6.9 × 10^10</td>
</tr>
<tr>
<td>2/88</td>
<td>70</td>
<td>1.1</td>
<td>22</td>
<td>4.2 × 10^6</td>
</tr>
<tr>
<td>2/89</td>
<td>40</td>
<td>1.2</td>
<td>24</td>
<td>1.7 × 10^7</td>
</tr>
<tr>
<td>11/89</td>
<td>50</td>
<td>0.8</td>
<td>16</td>
<td>6.6 × 10^4</td>
</tr>
<tr>
<td>13/89</td>
<td>60</td>
<td>1.8</td>
<td>36</td>
<td>6.9 × 10^10</td>
</tr>
<tr>
<td>18/89</td>
<td>85</td>
<td>1.2</td>
<td>24</td>
<td>1.7 × 10^7</td>
</tr>
<tr>
<td>25/89</td>
<td>65</td>
<td>1.9</td>
<td>38</td>
<td>2.7 × 10^11</td>
</tr>
</tbody>
</table>

a Tumor diameter at time of operation in millimeters.
b Decrease in TRF length based on T1 samples.
c Assuming a loss of 50 base pairs/cell division.
results. For example, tumor 20/87 had lost about 0.7 kilobase pairs (sample T1), giving 14 cell division rounds, which seems unlikely, when taking into account a tumor diameter of 150 mm. One explanation for this finding could be the activation of telomerase in this tumor adding new TTAGGG sequences to the chromosome ends. The same phenomenon was seen in another tumor (11/89) with an estimated number of 16 cell divisions, although the discrepancy between size and cell number was not as remarkable as in tumor 20/87. Another indication of telomerase activation in a subset of neoplastic cells is the peak TRF values near the peak of normal tissue in some of the samples.

When making such a comparison between the estimated number of cells in a tumor and the tumor size there are some obvious problems. We do not know the true telomere reduction per cell division in the tumors studied. If the reduction in telomere length per cell division is, e.g., 25 or 75 base pairs it will make a great difference when estimating total cell numbers. Furthermore, we cannot exclude the possibility that specific tumors have individual characteristics for this feature. In most solid tumors there is a continuous cell loss due to various factors, and depending on the cell loss fraction the accuracy of the estimated total tumor cell number will differ. However, if the tumor cell loss parameter were to be included in our theoretical considerations it would strengthen our suggestion of telomerase activation in some tumors. Since it is impossible to determine the moment when the tumorigenic process started, we cannot give an absolute value of the telomere length of the corresponding normal cells at that moment and consequently the "true" TRF reduction is unknown for the tumors.

Most of the studied samples gave reduced signal intensities, i.e., TRF volumes. This is in line with previous published data from other tumors (7, 8). However, there were some exceptions, like tumor 11/89, which showed a loss of around 0.8 kilobase pairs (mean TRF), but signal intensities in all three tumor samples were significantly over 100%. Furthermore, the T1 sample in tumor 2/89 showed a TRF reduction of 1.2 kilobase pairs, although there was an obvious increase in TRF volume. In these cases an activation of telomerase might also have occurred. An explanation for the remarkable increase in intensity, concomitant with a decrease in TRF length, might be that the activated telomerase has added TTAGGG units to chromosomes with telomeres that are extremely short or are absent from some chromosomes. This idea is supported by another study, where new TTAGGG repeats were added to a truncated chromosome in a case of thalassemia (17).

The TRF profile of the tumors showed two intrasample peaks detectable in half of the cases. This phenomenon has also been described in other malignancies (7, 8). One explanation has been that one peak represents an admixture of normal cells. Since various tissues have different TRF lengths depending on proliferative activity, a reasonable explanation could be that one peak was derived from, for example, fibroblasts, endothelial cells, or tumor-infiltrating lymphocytes. The volume of these "close to normal" peaks was as a rule so big that contamination of normal cells seemed less likely, based on the histopathological examination of the tumors. However, such a possibility cannot be ruled out. In a majority of the cases the peaks were too far displaced from the normal peak value to be representative of normal cells. We suggest that the finding of two peaks in a sample might be due to the coexistence of two major clones of tumor cells with different mean telomere lengths.

Our results showed intratumoral heterogeneity in peak TRF values in 70% of the tumors. One reasonable explanation for this might be that a subset of malignant cells will leave active cell cycle and enter a resting phase. Meanwhile, subclones of tumor cells continue to grow with accompanying gradual loss of telomeric sequences. We know from in vivo labeling of renal cell carcinomas with the thymidine analogue iododeoxyuridine that a small fraction of cells are in active S phase, indicating a significant fraction of resting cells. Another possible explanation for the existence of two peak values might be the hypothesis of multistep tumor progression through the accumulation of chromosomal aberrations (18), which can give rise to various clones with different growth properties. The found heterogeneity is supported by previous studies of renal cell carcinoma, where we have shown, by flow cytometry and at the molecular genetic level (13, 14), considerable intratumoral heterogeneity in DNA content.

In summary, our results demonstrate a shortening of telomeres together with an obvious intrasample/intratumor heterogeneity in peak TRF values. Although there are only indications, our study points out the possibility of activation of telomerase in a subset of the tumors investigated. In order to better understand the tumor biology of telomeres and telomerase as well as their clinical significance, future studies are needed to establish whether in vivo activation of telomerase occurs; such investigations have been initiated in our laboratory.

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