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Augmented telomerase activity, reduced telomere length and the presence of alternative lengthening of telomere in renal cell carcinoma: Plausible predictive and diagnostic markers

Deeksha Pal [a], Ujjawal Sharma [a], Ragini Khajuria [a], Shrawan Kumar Singh [b], Nandita Kakkar [c], Rajendra Prasad [a,*]

[a] Department of Biochemistry, PGIMER, Chandigarh, India
[b] Department of Urology, PGIMER, Chandigarh, India
[c] Department of Histopathology, PGIMER, Chandigarh, India

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ABSTRACT

In this study, we analyzed 100 cases of renal cell carcinoma (RCC) for telomerase activity, telomere length and alternative lengthening of telomeres (ALT) using the TRAP assay, TeloTTAGGG assay kit and immunohistochemical analysis of ALT associated promyelocytic leukemia (PML) bodies respectively. A significantly higher ($P = 0.000$) telomerase activity was observed in 81 cases of RCC which was correlated with clinicopathological features of tumor for instance, stage ($P = 0.008$) and grades ($P = 0.000$) but not with the subtypes of RCC ($P = 0.355$). Notwithstanding, no correlation was found between telomerase activity and subtypes of RCC. Strikingly, the telomere length was found to be significantly shorter in RCC ($P = 0.000$) to that of corresponding normal renal tissues and it is well correlated with grades ($P = 0.016$) but not with stages ($P = 0.202$) and subtypes ($P = 0.669$) of RCC. In this study, telomere length was also negatively correlated with the age of patients ($r^2 = 0.528$; $P = 0.000$) which supports the notion that it could be used as a marker for biological aging. ALT associated PML bodies containing PML protein was found in telomerase negative cases of RCC. It suggests the presence of an ALT pathway mechanism to maintain the telomere length in telomerase negative RCC tissues which was associated with high stages of RCC, suggesting a prevalent mechanism for telomere maintenance in high stages.
In conclusion, the telomerase activity and telomere length can be used as a diagnostic as well as a predictive marker in RCC. The prevalence of ALT mechanism in high stages of RCC is warranted for the development of anti-ALT inhibitors along with telomerase inhibitor against RCC as a therapeutic approach.

1. Introduction

Renal cell carcinoma (RCC) is the most prevalent and most lethal cancer of the kidney which accounts for approximately 3% of adult malignancies (Pal et al., 2014). A substantial gain in its incidence has been reported during the last decades (Siegel et al., 2014). Histopathologically, about 80% of RCCs are the clear cell type and the rest of the RCC subtypes include papillary, chromophobe & collecting duct. The clinical diagnosis of RCC is often confirmed by imaging studies, including X-ray and computed tomography but the possible existence of benign renal tumor is a serious challenge to the diagnosis. Importantly, RCC is one of the most therapy resistant cancers. It responds very poorly or not at all to chemotherapy and hormonal and radiation therapies (Walsh et al., 2002). It is notable that a better understanding of RCC tumor biology at the molecular level is utmost important to improve current diagnosis, prognosis and treatment of RCC.

One of the hallmarks of cancer is unlimited proliferation capacity, which is strictly associated with the ability to maintain telomeres through the activation of telomerase (Xu et al., 2013). Telomeres consist of 4 to 15 kb of the repetitive hexamer DNA sequence (TTAGGG) at the ends of chromosomes and undergo progressive shortening approximately 50–100 bp with each round of cell division (Blackburn, 1991). The repetitive noncoding telomeric repeats function as a buffer zone preventing the adjacent coding region of the genome from erosion by the telomeric DNA shortening.

Telomerase, or telomere terminal transferase, is a ribonucleoprotein that involves in the de novo synthesis and elongation of telomeric repeats onto chromosomal ends by adding (TTAGGG)n repeats (Blackburn, 1991). Functionally, immortal germ cell lines express telomerase and maintain sufficient telomeric repeats, whereas most human somatic cells are unable to acquire telomerase activity in successive cell culture and become senescent (Allsopp et al., 1995).

Abbreviations: RCC, renal cell carcinoma; ALT, alternative lengthening of telomeres; PML, promyelocytic leukemia; TRAP, telomere repeat amplification protocol.

* Corresponding author.
E-mail address: fateh1977@yahoo.com (R. Prasad).

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Stabilization of telomeres by telomerase reactivation appears to be concomitant with attainment of immortality which is likely to be necessary for infinite tumor growth. Most of the cancer cells maintain the telomere length with telomerase (Kim et al., 1994). Fewer yet significant numbers of tumor cells bypass the end replication in a telomerase independent manner, the process of which is called alternative lengthening of telomeres (ALT). Several lines of evidences indicate that an ALT involves a recombination based mechanism (Dunham et al., 2000) and characteristics of ALT cells include long and heterogeneous telomeres and subnuclear structure, termed as ALT associated promyelocyctic leukemia (PML) bodies (APBs). These structures contain telomeric DNA, telomere specific binding proteins (TRF1 & TRF2) and proteins involved in DNA replication and recombination (Cesare and Reddel, 2010). However, in few cases of cancers telomere maintenance mechanism is still not known (Ulaner et al., 2003).

There are mounting evidences for the existence of a significant relationship between telomeres, telomerase and cancer and cancer mortality (Artandi and DePinho, 2010; Willeit et al., 2010; Shay and Wright, 2011). There are only few reports available in the literature on evaluation of telomerase activity (Yoshida et al., 1998; Fujioka et al., 2000) as well as telomere length in RCC (Mehle et al., 1994; Dahse et al., 1999). Approximately, 85% of all human tumors have telomerase activity (Hrdlicková et al., 2012) and specific cancer subset exhibits the ALT phenotype, a telomerase independent telomere maintenance mechanism (Nguyen et al., 2013). Nevertheless, the presence of ALT in human RCC has not been studied extensively. Moreover, assessments of telomerase activity, telomere length and ALT as well as their association with grades and stages have not been evaluated in RCC patients. In view of these facts, a comprehensive study was extended to interpret the status of telomerase activity, telomere length and ALT in RCC progression, diagnosis and the future treatment by targeting telomerase/telomere or ALT pathway.

2. Materials and methods

2.1. Tissue specimens

The present study was approved by the institute ethics committee and informed consent was obtained from patients. Following nephrectomy, tissue samples were taken from the tumor and grossly normal renal parenchyma separately. The samples were snap frozen in liquid nitrogen and stored at −80 °C till further use. Grading of tumors was done by the Fuhrman grading system (Fuhrman et al., 1982).

2.2. Telomerase activity by telomere repeat amplification protocol (TRAP) assay

The tissue samples (50–100 mg) were homogenized and grinded after freezing in liquid nitrogen. The finely minced tissue was then extracted with lysis solution from the TeloTAGGG Telomerase PCR ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, USA) following the manufacturer's recommendations. The supernatant was transferred to a fresh tube and the total protein concentration was determined by the Lowry method (Lowry et al., 1951). Subsequently, TRAP reaction was carried out with 5 μg of total protein extract in a final volume of 50 μl, starting with the primer elongation step for 30 min at 25 °C, followed by telomerase inactivation for 5 min at 94 °C and 33 PCR cycles with denaturing for 30 s (94 °C), annealing for 30 s (50 °C), and polymerization for 90 s (72 °C). A 5 μl aliquot of the PCR product was denatured, hybridized to a DIG-labeled telomeric repeat-specific probe and immobilized onto a streptavidin-coated microtiter plate via the biotin-labeled synthetic PI-TS primer used for PCR. The amount of immobilized PCR product was determined with a peroxidase-conjugated anti-DIG antibody followed by a 3,3′,5,5'-tetramethylbenzidine (TMB) substrate color reaction. Absorbance was measured at 450 nm (reference wavelength, 650 nm) using an ELISA reader. Negative controls were prepared by incubating the total protein extract for 20 min at 85 °C. The ELISA was performed in triplicate. The relative telomerase activities (RTA) of different samples were calculated by-

$$\mathrm{RTA} = \frac{(\mathrm{AS}-\mathrm{AS,O})/\mathrm{AS,IS}}{(\mathrm{ATS8}-\mathrm{ATS8,0})/\mathrm{ATS8,IS}} \times 100$$

where

AS	absorbance of the sample
AS,0	absorbance of heat treated samples
AS,IS	absorbance of internal standard of the sample (IS)
ATS8	absorbance of control template (TS80)
ATS8,0	absorbance of lysis buffer
ATS8,IS	absorbance of internal standard (IS) of the control template (TS8).

2.3. Telomere restriction fragment length analysis

The genomic DNA was extracted using the standard method, with proteinase K treatment and phenol/chloroform extraction from 25 to 50 mg of the frozen tissue (Ausubel et al., 2002). Telomere length was assessed by measuring the telomere restriction fragments using the TeloTTAGGG telomere length assay kit (Roche Diagnostics, Indianapolis, USA) by following manufacturer's instructions. After digestion of the genomic DNA with Hinf1 and Rsa1 restriction enzymes, fragments were separated on a 0.8% agarose gel. The DNA was then transferred onto the nylon membrane (Roche Diagnostics, Indianpolis, USA) by Southern blotting and the blotted DNA fragments were hybridized with digoxigenin-labeled probe specific for telomeric repeats and incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphate. Finally, the immobilized telomere probe was visualized by metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. The average TRF length was calculated by the following formula given in the kit.

$$\mathrm{TRF} = \frac{\sum(\mathrm{OD_i})}{\sum(\mathrm{OD_i}/\mathrm{L_i})}$$

where,

OD_i	chemiluminescent signal
L_i	length of TRF at position i.

2.4. Immunohistochemical analysis of ALT associated PML bodies

Goat polyclonal IgG antibody directed against the N-terminus of the human PML protein (Santa Cruz, USA) was used for immunostaining at 1:100 dilutions (Sharma et al., 2014). Briefly, 5 μm tissue sections were cut, deparaffinized in xylene followed by rehydration in 100%, 95% and 70% ethanol. Antigen retrieval was carried out by boiling the slides in 10 mM citrate buffer, pH 6.0 for 15 min. Slides were then treated with blocking serum for 10 min, after which they were incubated with primary antibody at 4 °C overnight in a humid chamber, followed by incubation with HRP anti-goat IgG (1:100) secondary antibody overnight in a humid chamber (Bangalore Geni, India). Chromogen detection was performed with diaminobenzidine (DAKO Corp., Carpinteria, CA) solution (0.5 ml of stock DAB in 4.5 ml of Tris buffer with 20 μl of hydrogen peroxide). Slides were counterstained with hematoxylin and photographed.

2.5. Statistical analysis

Statistical evaluation was performed using SPSS program (version 20.0; SPSS Inc., Chicago, IL). Differences in mean telomerase activity as well as relative telomere length between two groups were evaluated using Mann–Whitney and between more than two groups were