Expression of Telomere Binding Proteins (RAP1 and POT1) in Renal Cell Carcinoma and Their Correlation with Clinicopathological Parameters

Deeksha Pal^{1,2} · Shrawan Kumar Singh³ · Nandita Kakkar⁴ · Rajendra Prasad¹

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Abstract Telomere stability is indispensable for continuous proliferation of cells. Telomere structure is maintained by group of six proteins termed as shelterin. RAP1 and POT1 proteins are significant members of shelterin complex. Expression of RAP1 and POT1 are crucial for telomere maintenance and hence uncontrolled division of cells. Notably, expression of RAP1 and POT1 is unknown in renal cell carcinoma (RCC). In view of these facts, the present study was initiated to investigate the expression of RAP1 and POT1 in RCC and their relationships with clinicopathological features. In total 65 histopathologically confirmed RCC cases and their adjacent normal renal parenchyma were analyzed for gene expression. The mRNA expression of telomere binding proteins RAP1 and POT1 were measured using RT-PCR. Expression of RAP1 was observed to be significantly increased in tumour tissues as compared to corresponding normal renal tissues ($P = 0.004$). The gene expression of RAP1 was documented to be related to grades of RCC ($P = 0.002$) and subtypes of RCC ($P = 0.01$). Although, POT1 expression was up-regulated in RCC tissue, however it was not statistically significant. Also, POT1 expression was not related to grades, stages and subtypes of RCC. This is the first study which shows correlation RAP1 with grades and subtypes of RCC.

Keywords Kidney · Cancer · Genomics · RAP1 · POT1 · Correlation

Introduction

Renal cell carcinoma (RCC) is the most common kidney cancer in adults which constitute ~90 % of all primary adult renal tumors [1]. As, diagnosis of RCC often occurs at advance disease stage hence survival of patient is very poor. The exact cause of RCC carcinogenesis is still not well known. Carcinogenesis is a multistep process that involves chromosome instability which leads to multiple mutations involving activation of oncogenes, loss of tumor suppressor genes. Telomere dysfunction is one of the main probable reasons of chromosome instability [2, 3]. Telomeres are specialized nucleoprotein complexes that are located at the ends of chromosomes [4]. Human telomeres are composed of up to 10–15 kb hexanucleotide TTAGGG repeats and associated telomere binding proteins that generate protective telomere-specific complexes [5, 6]. In higher eukaryotes, a set of six telomere-specific proteins termed “shelterin” has been identified, which are central to telomere maintenance [7]. The telosome/shelterin complex consists of important regulatory telomere binding proteins to include tripeptidyl peptidase1 (TPP1), protection of telomere 1 (POT1), TRF1 interacting nuclear factor 2 (TIN2), repressor activator protein (RAP1), telomere repeat binding factor 1 (TRF1) and TRF2 [8].

Telomere binding proteins TRF1, TRF2 and POT1 bind directly to telomeric repeats and TIN2, TPP1 and RAP1 are interconnected to these proteins thus form a functional complex which caps telomeric ends [6]. POT1 specifically binds to the overhang single-stranded

✉ Rajendra Prasad
fateh1977@yahoo.com

¹ Department of Biochemistry, PGIMER, Chandigarh, India

² Department of Urology, University of Louisville, Louisville, KY, USA

³ Department of Urology, PGIMER, Chandigarh, India

⁴ Department of Histopathology, PGIMER, Chandigarh, India

telomeric DNA and shields it from degradation. POT1 recruits telomerase to the single-strand telomeric overhang and thus can act as a telomerase-dependent, positive regulator of telomere length [9, 10]. RAP1 forms a complex with TRF2 and affects the length and heterogeneity of human telomeres and thus play a role in telomere function and end protection [11]. Any changes in telomere binding proteins expressions can lead to telomere dysfunction. Oncogenesis research is now focusing on the shelterin complex. Several studies have shown the association or role of shelterin proteins in various cancers viz. lymphocytic leukemia [12], squamous cell carcinoma [13], non-small cell lung cancer [14] and breast cancer [15]. However, the expressions of telomere binding proteins and their association with clinicopathological parameters in RCC have not been well studied.

In the present study, mRNA expression of the genes encoding the proteins POT1 and RAP1 in RCC and their adjacent normal renal parenchyma were evaluated. The association between expressions of RAP 1 and POT1 and the clinicopathological findings were also assessed.

Materials and Methods

Tissue Samples

The study was approved by the Institute ethics committee and informed consent was taken from all patients. RCC tissue and paired adjacent normal renal parenchyma were obtained from 65 RCC patients who underwent radical nephrectomy. All the tissue samples were immediately snap-frozen and stored at -80°C till used further. Specimens were confirmed by pathological examination. Tumor staging and grading of clear cell RCC type was performed according to TNM staging [16] and Fuhrman grading [17] respectively.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from both tumor as well as normal tissue with Trizol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. The concentration was measured by spectrophotometry and formaldehyde denatured agarose gel electrophoresis was used for quality examination. Before reverse transcription, genomic DNA contamination was eliminated by treating $1\text{ }\mu\text{g}$ RNA with 1 U DNase I (Fermentas) for 30 min at 37°C and then heat-inactivated at 65°C for 10 min. Total RNA was reverse transcribed into cDNA by using the SuperscriptIII first-strand synthesis system (Invitrogen, USA) as per manufacturer's protocol.

The cDNA samples were stored immediately at -20°C for succeeding real time PCR analysis.

Quantitative Real-Time PCR

Real-time analysis was performed on 7300 RT-PCR system (Roche, Indianapolis, IN) using the SYBR Green PCR Master Mix (Roche Indianapolis, IN). The oligonucleotide sequences of the primers were as follows:

RAP1 Forward-5' GCCACCCGGGAGTTTGA 3'
Reverse-5' GGGTGGATCATCATCACACATAGT-3'
POT1 Forward 5' CAGAACCTGACGACAGCTTTCC 3'
Reverse 5' GCACATAGTGGTGTCTCTCCA 3'
 β -actin Forward 5' CGAGCGCGGCTACAGCTT 3'
Reverse 5' TCCTTAATGTACGCACGATTT 3'

The cycles were programmed for 95°C for a 5 min initial step, then 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. At the same time no-template controls was also performed in each experiment and no signals were detected in these. The average threshold cycle for each sample was determined from triplicate reactions and the expression level was normalized to β -actin. The relative amount of mRNA was calculated by $\Delta\Delta C_t$ method using the following equation [18].

Relative expression : $2^{-(\text{Tumor}\Delta C_t - \text{Normal}\Delta C_t)}$

where ΔC_t = average C_t (RAP1 or POT1) – average C_t (β actin).

Statistical Analysis

Statistical analysis was performed using the SPSS 20.0 software package (SPSS, Chicago, IL, USA). The statistical significance of the differences in mRNA expression levels between two groups and more than two groups were analyzed by using Student's *t* test and an ANOVA respectively. The correlation between the expression of RAP1 or POT1 and various clinicopathological features was calculated using Spearman's rank correlation test. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Patient's Characteristics

A total of 65 cases of histopathologically proven RCC were included in this study. The mean age of the patients in this study was 54 ± 14.6 years. Low stage includes stage 1 and 2 whereas; high stage includes stage 3 and 4. Most of the patients had low stage clear cell RCC (75.0 %), in

Table 1 Clinical characteristic of the patients

Patients (n)	65
Gender, n (%)	
Male	47 (72.3)
Female	18 (27.7)
Age (years), mean $\pm$ SD	54 $\pm$ 14.6
BMI, mean $\pm$ SD	25.67 $\pm$ 0.364
Commonest presenting complaints, n (%)	
Hematuria	36 (55.4)
Flank pain	22 (33.8)
Both	18 (27.7)
Incidental radiological examination	7 (10.8)
Histologic subtypes, n (%)	
Clear cell RCC	44 (67.7)
Papillary	12 (18.5)
Sarcomatoid	6 (9.2)
Chromophobe	3 (4.6)
Pathologic stages (clear cell), n (%)	
T1	23 (52.3)
T2	10 (22.7)
T3	9 (20.5)
T4	2 (4.5)
Fuhrman grades (Clear Cell), n (%)	
I	9 (20.5)
II	21 (47.7)
III	10 (22.7)
IV	4 (9.1)

SD standard deviation

comparison to the high stages clear cell RCC (25.0 %). Grade 1 and 2 specimens were included in low grade, whereas grade 3 and 4 specimens were included in high grade. Most of the patients were of low grades (68.2 %). Table 1 shows the detail clinical characteristics of all the patients.

RAP1 and POT1 Gene Expressions in RCC

We observed a significantly higher expression of RAP1 mRNA in RCC tissues in comparison to that in adjacent normal renal tissues (fold change median value, 4; $P = 0.004$; Fig. 1a). Also, a higher expression of POT1 mRNA was observed in RCC tissues as compared to adjacent normal renal parenchyma (fold change median value, 2.02; $P = 0.05$; Fig. 1b).

Correlation of RAP1 and POT1 Gene Expression with Clinicopathological Parameters

Additionally, an attempt was made to correlate the RAP1 mRNA expressions with various clinicopathological parameters. The RAP1 mRNA expression was significantly

declined in clear cell RCC with high grades in comparison to low grade clear cell RCC (low grade vs high grade; 6.60 vs 2.57; $P = 0.002$; Fig. 2a). Further, a significant difference was noticed in the RAP1 mRNA expression among different subtypes of RCC (Fold change: Median value; ccRCC; 4.75, pRCC; 2.00, ChRCC; 9.00, srCC; 1.8; $P = 0.01$; Fig. 2b). However, no significant change in RAP1 mRNA expression was observed between low stage and high stage of clear cell RCC ($P = 0.673$). Conversely, no correlation could be established between POT1 mRNA expression and grades ($P = 0.562$), stages ($P = 0.756$) or subtypes of RCC ($P = 0.695$).

Discussion

RAP1 and POT1, the telomere associated proteins are part of telomere structure and have an essential role in telomere maintenance. POT1 is involved in directly regulating telomere length. It is homologous in budding and fission yeasts where it is known to regulate the ability of telomerase to elongate telomeres [19]. Studies have shown that human POT1 is the only protein of shelterin complex that binds to telomeric ssDNA [9] which is a substrate of telomerase. Hence, binding of telomerase to its substrate can be modulated by POT1 at least in vitro. Overall, POT1, along with both telomeric ssDNA and TRF2, is critical for regulating telomere length [20]. In this study, POT1 expression was found to be higher in RCC as compared to normal renal parenchyma. But, increased in POT1 expression was not statistically significant. Hence, this increased POT1 expression may be required for maintaining the telomere function in RCC.

Earlier, it has been shown that POT1 expression may be persuaded by tumor stage and telomere length. Previous study in gastric cancer indicated that POT1 is down-regulated in stage I/II whereas it is upregulated in stage III/IV [21]. However, we did not find any correlation between POT1 expressions with stages of RCC. Human RAP1 (hRAP1) is employed to the telomere by TRF2 [22]. It was shown that hRAP1 was shown to have similar functions to its yeast counterpart. Budding yeast RAP1 plays a role in transcription, telomere length regulation, and end capping. Thus, it plays a role in telomere maintenance. In our present study, a significantly higher expression of RAP1 was observed in tumor tissue than in normal renal tissues. This increase in RAP1 expression indicates the protective role of RAP1 in telomere protection in RCC. Previously, it was observed that RAP1 alone suppresses non homologous end joining in human tissue extracts and homologous recombination in mouse cells [23]. Additionally, RAP1 expression was found to be inversely correlated with tumor grades in present study. Its expression was high in low grade tumors and low in high grade tumors.

Fig. 1 Box plot diagram shows the gene expression of **a** RAP 1 and **b** POT 1 relative to control. The extreme values were noted as open circles and star their sample number mentioned against them. One

sample *t* test was used for statistical analysis. $P < 0.05$ was considered as significant. *** $P < 0.001$

Fig. 2 The RAP1 mRNA expression level in different **a** grades and **b** types of RCC was determined by quantitative real time PCR. The bottom and top edges of the box located at the 25th and 75th percentiles respectively of the sample. The centre horizontal lines are drawn at the median of the sample. The extreme values were noted as

stars their sample number mentioned against them. mRNA level of β -actin was used to normalize RAP1 gene expression. Statistical analysis was done by Mann–Whitney and Kruskal–Wallis test respectively. *** $P < 0.001$

Tumor grade is a sign of the severity of the malignancy. It refers to the resemblance of the tumor cells to the normal cells of the same tissue type. Low grade cells resemble to normal cells and tend to grow and multiply slowly, whereas high grade tumors grow rapidly. Therefore, inverse relation of RAP1 suggested that its expression might have a protective role in RCC progression. However, there was no difference in RAP1 expression among different stages of clear cell RCC.

Our findings are consistent with the previous study where authors have shown that RAP1 expression is associated with tumor grade and overall survival of the lung cancer patients [14]. Nevertheless, their data strongly support that expression of RAP1 is a promising prognosis marker for lung cancer. In another study by Chen et al. [24] examined the mRNA and protein level expression of RAP1 at different stages of oral cavity squamous cell carcinoma (OSCC). They have reported strong RAP1 expression as a

significant prognostic marker and predictor of aggressive OCSCC. In the present study RAP1 expression was significantly correlated with different subtypes of RCC. This observation supports RAP1 expression as a possible marker of clinical behavior of RCC. However, still little is known about the function, role and mechanism of RAP1 in maintenance of telomeres. It has also shown that depletion of endogenous hRAP1 by small interference RNA leads to longer telomeres and over-expression of a dominant negative form of human RAP1 extends telomeres [25]. The precise role and functions of RAP1 and POT1 are largely unknown in cancer. But, it is evident that the coordinated interaction of these proteins with telomeric DNA is essential to maintain telomere stability. Also, damage in structural components of telomere leads to telomere associated DNA damage response and genome instability [26].

In conclusion, this is the first study exploring expressions of RAP1 and POT1 in different grades, stages and subtypes of RCC. Further, a correlation was established between RAP1 expression with grades and subtypes of RCC. However, an extensive study is required to elucidate the role of RAP1 and POT1 in carcinogenesis of RCC.

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Compliance with Ethical Standards

Conflict of interest None.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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