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ANATOMICAL PATHOLOGY

Human telomerase reverse transcriptase protein expression predicts tumour aggressiveness and survival in patients with clear cell renal cell carcinoma

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Summary

Human telomerase reverse transcriptase (hTERT) is an active component of telomerase and responsible for its catalytic activity, associated with cell proliferation and differentiation. For the first time, the present study was conducted to evaluate the expression and prognostic significance of hTERT in different histological subtypes of renal cell carcinoma (RCC). Expression of hTERT was examined in 176 well-defined renal tumour samples including clear cell RCCs (ccRCCs), papillary and chromophobe RCCs using immunohistochemistry on tissue microarrays. The association between hTERT expression and clinicopathological parameters as well as survival outcomes were then analysed. There was a statistically significant difference in terms of hTERT expression among various RCC subtypes. In ccRCC, increased expression of hTERT was significantly associated with advanced stage, higher grade, presence of microvascular invasion, lymph node invasion, and metastasis. Moreover, in the multivariate analysis, tumour stage and tumour size were independent predictors of the disease-specific survival (DSS). Additionally, expression of hTERT was found to be a significant predictor of worse DSS ($p = 0.012$) in the univariate analysis. In papillary carcinoma samples (type I and II), significant association was detected between hTERT expression and the tumour stage ($p = 0.010$, $p = 0.050$), respectively. In chromophobe RCC, no significant association was detected between expression of hTERT and clinicopathological parameters and survival data. We showed that hTERT protein expression was associated with more aggressive tumour behaviour and more advanced disease in ccRCC patients. Also, hTERT may be a novel poor prognostic indicator of DSS, if the patients are followed for more prolonged time periods.

Key words: hTERT; protein expression; renal cell carcinoma (RCC); clear cell renal cell carcinoma (ccRCC); tissue microarray (TMA).

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney (80–90%), accounting for a total of 2–3% of adult malignancies, and is the most fatal urological cancer.[1] RCC is classified into several subtypes: the most common subtype is clear cell renal cell carcinoma (ccRCC), representing 75–80% of primary renal malignancies, followed by papillary and chromophobe RCC accounting for 10–15% and 5% of renal cancers, respectively.[2] It is estimated that there will be 65,340 new cases of RCC in the United States and 14,970 related deaths in 2018.[3] Despite the recent advances in the diagnosis and treatment of RCC such as using tyrosine kinase and mTOR kinase inhibitors,[4,5] up to 30% of RCC patients present with distant metastatic disease and 20–40% develop recurrent disease after surgery; therefore, RCC is still one of the deadliest forms of malignancies with a continued increase in incidence.[6]

Human telomerase is a ribonucleoprotein enzyme consisting of two main components: catalytic component, human telomerase reverse-transcriptase (hTERT) and human telomerase RNA molecule (hTR), in addition to telomerase-associated proteins hTEP1, p23, Hsp90, and dyskerin. Telomerase adds the telomere repeat sequence (TTAGGG)n to the 3′ end of telomeres using its RNA as a template and hTERT.[7] Evidence shows that telomerase is expressed in 85% of malignancies, and the level of its activity is higher in advanced and metastatic tumours. While it is absent or barely detectable in most human normal somatic cells and germ cells, stem cells and cardiovascular cells express significant detectable levels of telomerase.[8–10]

A previous study has shown that hTERT, an active component of telomerase and responsible for its catalytic

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activity, has other functions unrelated to its reverse transcriptase activity, such as increasing the anti-apoptotic capacity, enhancing DNA repair, maintaining stem cells and regulating gene expression.[11]

A number of clinical studies have been performed to evaluate the association between telomerase activity and clinicopathological parameters in renal cancer showing that telomerase activity level correlates with progression of RCC.[12–14] However, we could not find any data regarding hTERT protein expression patterns and its association with clinicopathological parameters and survival in RCC patients.

In view of these facts, the present study was designed to investigate for the first time the expression levels and potential prognostic role of hTERT protein in a series of RCC clinical tumour samples, composed of clear cell, papillary, and chromophobe RCC using tissue microarray (TMA) method.

MATERIALS AND METHODS

Patient characteristics and tumour samples

A total of 202 paraffin embedded tissues from RCC clinical tumour samples were included in this study. These specimens were collected from the Hasheminejad Hospital, a major referral university-based urology-nephrology centre in Tehran, Iran, in the time period 2010–2015. All samples were collected from patients who had undergone radical nephrectomy and had no history of radiation therapy. These samples comprised various subtypes of RCC including clear cell, type I and II papillary, and chromophobe RCC. The haematoxylin and eosin (H&E) stained slides and medical archival records were retrieved to obtain clinicopathological parameters including age, gender, tumour size (maximum tumour diameter), tumour stage, and nucleolar grade. In addition, the presence of necrosis, distant metastasis, the involvement of regional lymph nodes, renal vein, sinus and pelvis, Gerota's fascia, and microvascular invasion (MVI) were recorded. Disease-specific survival (DSS) was defined as the time from radical nephrectomy to the date of death related to the patient's cancer. The stage was defined based on the pTNM classification for renal cell carcinomas.[15]

TMA construction

The renal TMAs were prepared as described previously.[16–18] In brief, H&E slides were examined and the most representative areas in different parts of the tumour were marked by two experienced pathologists (MA and MA). Microarray samples of 0.6 mm diameter were punched out from the selected regions of each donor block and precisely transferred into a new recipient paraffin block using Tissue Arrayer MiniCore (Alphelys, France). The validation study for TMAs revealed that despite the variability of antigen expression between the cores, analysis of each core was >90% accurate for estimating the staining pattern of the whole tissue section, whereas analysis of the two readable cores achieved >95% accuracy.[19] In the present study, three cores were punched and evaluated from each tumour and scored individually. Several previous validation studies have shown that three cores are highly representative for the whole sections.[20–23] In each TMA block, adjacent normal renal tissue samples (26 samples in total) were included to compare the expression model and the distribution of hTERT marker in a range of tissue specimens. Then, 4 μm sections were cut from the completed array blocks and transferred to adhesive slides. Next, TMA blocks were constructed in three copies for each specimen. The mean H-score value of three cores were calculated as final scores.

Immunohistochemistry (IHC) for hTERT protein expression

Briefly, all TMA sections were deparaffinised at 60°C for 20 min and dehydrated with graded alcohol. Endogenous peroxides and non-reactive staining were blocked with 3% H_2O_2 for 20 min at room temperature. After washing the tissue sections three times, antigen retrieval was performed by immersing the tissues in citrate buffer (pH 6.0) for 10 min in an autoclave. The tissue sections were incubated with primary antibody, anti-Telomerase reverse transcriptase antibody (ab183105, dilution: 1/500; Abcam, USA), overnight at 4°C. TMA slides were then incubated with anti-rabbit/anti-mouse Envision (Dako, Denmark) as a secondary antibody for 30 min. Staining patterns were visualised by exposure to 3, 3′-diaminobenzidine (DAB; Dako) followed by counterstaining with haematoxylin visualise antigen (Dako). Finally, the slides were dehydrated in alcohol, cleared in xylene (Dako), and mounted for examination. In each run of the experiment, human tonsillar tissue was used as a positive control; for a negative control, the primary antibody was replaced with Tris-buffered saline.

Evaluation of immunostaining

Immunostaining of telomerase reverse transcriptase (hTERT) was independently evaluated by two pathologists (MA and MA) who were blinded to the patients' outcome and pathological information. A consensus was achieved for all samples. The intensity of staining was scored by applying a semi-quantitative system, ranging from negative to strong as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was categorised according to the positive tumour cells as follows: Group 1, less than 25% positive cells; Group 2, 25–50% positive cells; Group 3, 51–75% positive cells; and Group 4, more than 75% positive cells. To compare all the available data, we assigned an overall histochemical score (H-score) to each case by multiplying the intensity score by the percentage of positive cells, which yielded a range from 0 to 300. In this study, median H-score was chosen to categorise samples as with high or low telomerase reverse transcriptase expression.

Statistical analysis

Data were analysed using the statistical software SPSS (version 20.0; IBM Corp, USA). We reported the categorical data by N (%), valid percent and quantitative data as follows, mean (SD) and median (Q1, Q3). hTERT expression in ccRCC, papillary (type I and II), and chromophobe RCC samples was compared using Kruskal–Wallis and Mann–Whitney *U* tests, for pairwise comparison between groups. Moreover, Pearson's chi-square and Spearman's correlation tests were used to analyse the significance of association and correlation between hTERT expression and clinicopathological parameters. Disease-specific survival (DSS) curves were drawn using the Kaplan–Meier method and log-rank test was used to compare the estimated curves between groups. The Cox proportional hazards regression model was applied to determine which variables affected DSS. Variables that significantly affected survival in univariate analysis were included in multivariable analyses. A p value of ≤ 0.05 was considered statistically significant.

Ethical approval and consent

This study was approved by the Iran University of Medical Sciences Human Research Ethics Committee in Iran (Ref no: IR.IUMS.REC1395.25153). All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual participants included in the study at the time of sample collection with routine consent forms.

RESULTS

Patients' characteristics

Of 202 cases, 176 RCC patients were evaluated, of which 113 (64.2%) were ccRCC, 12 (6.8%) type I, 20 (11.4%) type II papillary, and 31 (17.6%) chromophobe RCC. Tumour heterogeneity is a major concern when using TMA method. Therefore, some other studies have recommended at least two or three cores to be most efficient.[19,21] In this study, three cores were evaluated from each tumour. Due to missing one or two cores of each sample within the immunohistochemistry staining, 26 RCCs were excluded from the study, leaving 176 RCCs tissues for final scoring.

The study population consisted of 123 (69.9%) male and 53 (30.1%) female patients, with a male/female ratio of 2.3. The age variable followed a normal distribution; therefore,

mean age of patients was calculated as 55 years (SD = 13, range 25–82); 85 (48.3%) patients were younger than 55, and 91 (51.7%) were over 55 years old.

Tumour size ranged from 1 to 21 cm in largest diameter and tumours were classified into four groups: Group 1, 0–4 cm (34, 19.3%); Group 2, 4.1–7.0 cm (63, 35.8%); Group 3, 7.1–10.0 cm (40, 22.7%); and Group 4, >10.1 cm (39, 22.2%).

In this study, 90 (51.1%) patients had low nucleolar grade tumours (grade II), 48 (27.3%) had grade III tumours, and seven (4.0%) had high nucleolar grade tumours (grade IV). Moreover, 45 (25.6%) cases were stage I, 13 (7.4%) were stage II, 103 (58.5%) were stage III, and 15 (8.5%) were stage IV.

Regional lymph node involvement was found in 12 (6.8%) cases; 33 (18.8%) cases had MVI, 100 (56.8%) had renal sinus fat involvement, and 74 (42.0%) cases had tumour necrosis. Moreover, 37 (21.0%) cases showed subsequent metastasis during the follow-up period. Other less frequent sites of involvement were as follows: renal vein invasion (9, 5.1%), renal pelvis invasion (13, 7.4%), perirenal fat invasion (30, 17.0%), and Gerota's fascia invasion (4, 2.3%).

Table 1 Patients and pathological characteristics of various subtypes of RCC

Patients and tumour characteristics	Total samples *n* (%)	RCC			
		Clear cell *n* (%)	Papillary *n* (%)		Chromophobe *n* (%)
			Type I	Type II	
No. tumour samples	176	113 (64.2)	12 (6.8)	20 (11.4)	31 (17.6)
Mean age, years (range)					
≤Mean age	55 (25–82)	56 (25–82)	60 (43–76)	52 (25–73)	49 (27–76)
>Mean age	85 (48.3)	54 (47.8)	7 (58.3)	9 (45.0)	17 (54.8)
	91 (51.7)	59 (52.2)	5 (41.7)	11 (55.0)	14 (45.2)
Gender					
Male	123 (69.9)	79 (69.9)	12 (100.0)	14 (70.0)	18 (58.1)
Female	53 (30.1)	34 (30.1)	0 (0.0)	6 (30.0)	13 (41.9)
(Male/Female)	2.3	2.3	12.0	2.3	1.3
Tumour size (cm)					
0–4	34 (19.3)	25 (22.1)	2 (16.7)	4 (20.0)	3 (9.7)
4.1–7	63 (35.8)	40 (35.4)	4 (33.3)	6 (30.0)	13 (41.9)
7.1–10	40 (22.7)	28 (24.8)	3 (25.0)	6 (30.0)	4 (12.9)
>10.1	39 (22.2)	20 (17.7)	3 (25.0)	4 (20.0)	11 (35.5)
Nucleolar grade					
I	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
II	90 (51.1)	78 (69.0)	9 (75.0)	4 (20.0)	0 (0.0)
III	48 (27.3)	28 (24.8)	3 (25.0)	16 (80.0)	0 (0.0)
IV	7 (4.0)	7 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)
Primary tumour (PT) stage					
pT1	45 (25.6)	33 (29.2)	5 (41.7)	3 (15.0)	4 (12.9)
pT2	13 (7.4)	7 (6.2)	3 (25.0)	2 (10.0)	2 (6.5)
pT3	103 (58.5)	65 (57.5)	4 (33.3)	9 (45.0)	24 (77.4)
pT4	15 (8.5)	8 (7.1)	0 (0.0)	6 (30.0)	1 (3.2)
Microvascular invasion (MVI)					
Present	33 (18.8)	19 (16.8)	0 (0.0)	4 (20.0)	10 (32.3)
Absent	143 (81.3)	94 (83.2)	12 (100.0)	16 (80.0)	21 (67.7)
Lymph node invasion (LNI)					
Involved	12 (6.8)	8 (7.1)	11 (91.7)	4 (20.0)	0 (0.0)
None	161 (91.5)	103 (91.2)	1 (8.3)	16 (80.0)	31 (100.0)
Not identified	3 (1.7)	2 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)
Renal vein invasion					
Present	9 (5.1)	8 (7.1)	0 (0.0)	1 (5.0)	0 (0.0)
Absent	167 (94.9)	105 (92.9)	12 (100.0)	19 (95.0)	31 (100.0)
Tumour necrosis					
Present	74 (42.0)	40 (35.4)	8 (66.7)	16 (80.0)	10 (32.3)
Absent	101 (57.4)	73 (64.6)	4 (33.3)	4 (20.0)	20 (64.5)
Not identified	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.2)
Renal sinus fat invasion					
Present	100 (56.8)	60 (53.1)	2 (16.7)	15 (75.0)	22 (71.0)
Absent	76 (43.2)	53 (46.9)	10 (83.3)	5 (25.0)	9 (29.0)
Renal pelvis invasion					
Present	13 (7.4)	7 (6.2)	0 (0.0)	5 (25.0)	0 (0.0)
Absent	163 (92.6)	106 (93.8)	12 (100.0)	15 (75.0)	31 (100.0)
Perirenal fat invasion					
Present	30 (17.0)	19 (16.8)	1 (8.3)	4 (20.0)	6 (19.4)
Absent	146 (83.0)	94 (83.2)	11 (91.7)	16 (80.0)	25 (80.6)
Gerota's fascia invasion					
Present	4 (2.3)	4 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)
Absent	172 (97.7)	109 (96.5)	12 (100.0)	20 (100.0)	31 (100.0)
Distant metastasis					
Present	37 (21.0)	28 (24.8)	0 (0.0)	5 (25.0)	4 (12.9)
Absent	139 (79.0)	85 (75.2)	12 (100.0)	15 (75.0)	27 (87.1)

RCC, renal cell carcinoma.

The clinicopathological features of patients are summarised in Table 1 based on subtypes of RCC. It was agreed that chromophobe RCC should not be graded.[24]

Comparison of hTERT protein expression in RCC subtypes

hTERT was expressed with variable intensities in the nucleus and also partially in the cytoplasm in most of the RCC samples. Since the translocation of hTERT from cytoplasm to nucleus is crucial for the physiological activation of hTERT in maintaining the telomere length,[25,26] we focused only on nuclear hTERT expression and impact on the clinicopathological parameters of RCC samples in this study. Expression of hTERT was observed in the tubules and capsules of the normal renal parenchyma; however, the expression of this marker was much lower in the cytoplasm of the normal renal

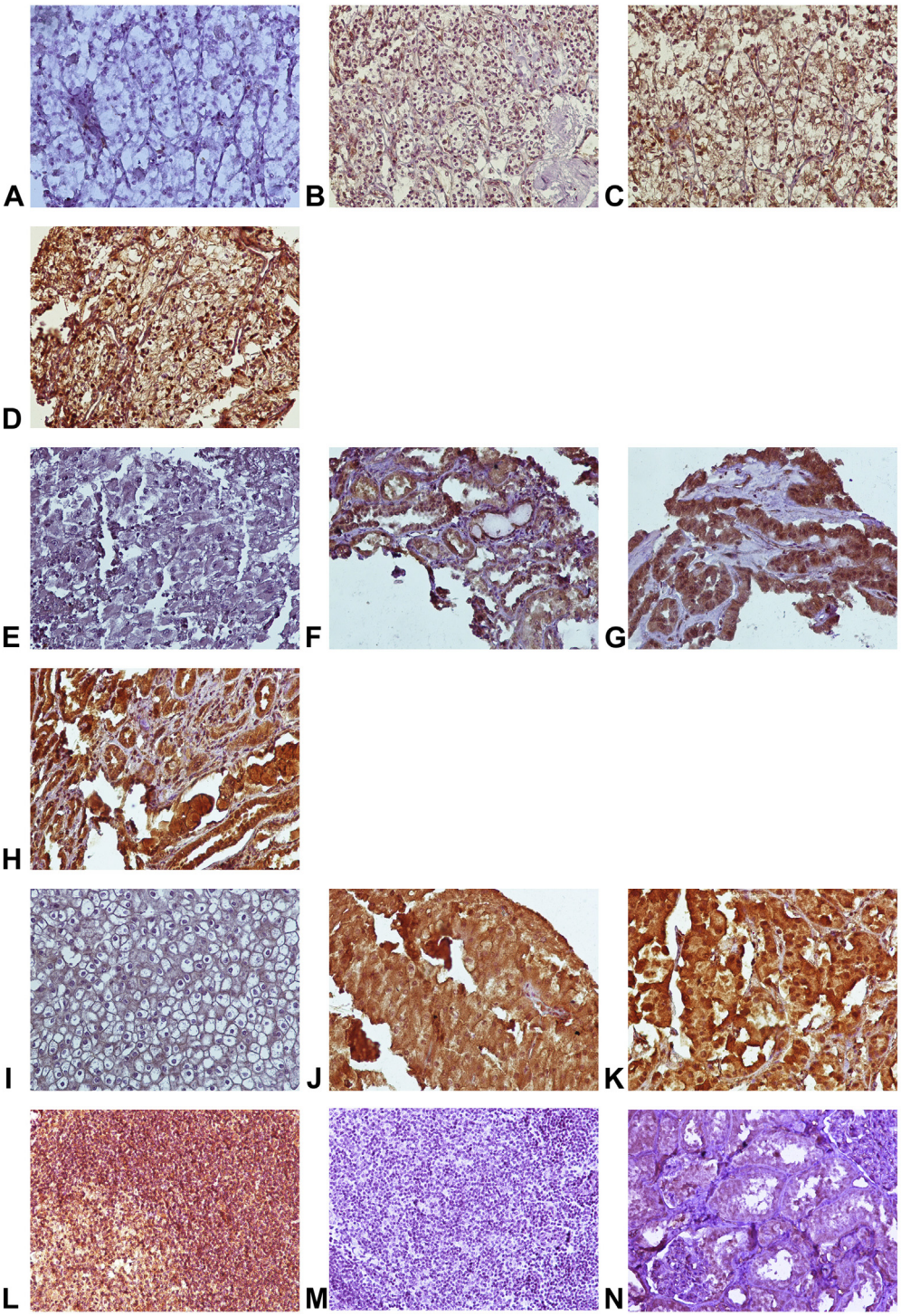

Fig. 1 Immunohistochemical analysis of hTERT expression in renal cell carcinoma (RCC) samples. hTERT expression in clear cell RCC: (A) 0, no intensity; (B) +1, weak; (C) +2, moderate; (D) +3, strong. hTERT expression in papillary RCC: (E) 0, no intensity: (F) +1, weak; (G) +2, moderate; (H) +3, strong. hTERT expression in chromophobe RCC: (I) 0, no intensity: (J) +1, weak; (K) +2, moderate. IHC staining of normal tonsil tissue as (L) positive and (M) negative controls and also (N) normal renal sample to compare the expression of the hTERT marker between renal tumours and normal renal samples.

Table 2 Human telomerase reverse transcriptase expression (intensity of staining, percentage of positive tumour cells, and H-score) in various subtypes of RCC

Scoring system	Total samples *n* (%)	RCC			
		Clear cell *n* (%)	Papillary (Type I and II) *n* (%)	Chromophobe *n* (%)	*p* value
Intensity of staining					
No staining (0)	4 (2.3)	1 (0.9)	1 (3.1)	2 (6.5)	**<0.001**
Weak (+1)	52 (29.5)	19 (16.8)	6 (18.8)	27 (87.1)	
Moderate (+2)	70 (39.8)	60 (53.1)	8 (25.0)	2 (6.5)	
Strong (+3)	50 (28.4)	33 (29.2)	17 (53.1)	0 (0.0)	
Percentage of positive cells					
<25%	38 (21.6)	20 (17.7)	2 (6.3)	16 (51.6)	**<0.001**
25–50%	40 (22.7)	27 (23.9)	3 (9.4)	10 (32.3)	
51–75%	23 (13.1)	15 (13.3)	3 (9.4)	5 (16.1)	
>75%	75 (42.6)	51 (45.1)	24 (75.0)	0 (0.0)	
H-score					
Low (≤140)	90 (51.1)	48 (42.5)	11 (34.4)	31 (0.0)	**<0.001**
High (>140)	86 (48.9)	65 (57.5)	21 (65.6)	0 (0.0)	
Total	176	113	32	31	

p value, Pearson's χ^2 test. Values in bold are statistically significant.
H-score, histological score; RCC, renal cell carcinoma.

cells compared to renal cancer tissues, and also nuclear staining was not observed in these cases. Strong nucleus staining was detected in human tonsil tissue as a positive control (Fig. 1).

In this study, there was no variability in the IHC staining in three cores from different parts of the tumour; however, mean H-score value of three cores was calculated as a final score.

Of 176 RCC samples stained for hTERT, four (2.3%) did not show any staining, whereas weak, moderate, and strong staining intensities were observed in 52 (29.5%), 70 (39.8%), and 50 (28.4%) cases, respectively. The expression of hTERT marker was classified into two groups as described earlier. The evaluation of hTERT expression based on the H-score values demonstrated that low expression of hTERT was found in 90 (51.5%) and high expression was found in 86 (48.9%) RCC samples. Pearson's χ^2 test showed a statistically significant association between hTERT expression and RCC subtypes ($p < 0.001$) (Table 2).

The non-parametric Kruskal–Wallis and Mann–Whitney *U* tests were used to compare differences between median of hTERT expressions among the three tumour subtypes. Results of the Kruskal–Wallis test indicated a statistically significant difference between the various levels of hTERT expression in different RCC subtypes ($p < 0.001$). Using the Mann–Whitney *U* test, a statistically significant difference in the median level of hTERT expression between ccRCC and chromophobe samples and also papillary (type I and II) and chromophobe RCC was found ($p < 0.001$) (Fig. 2).

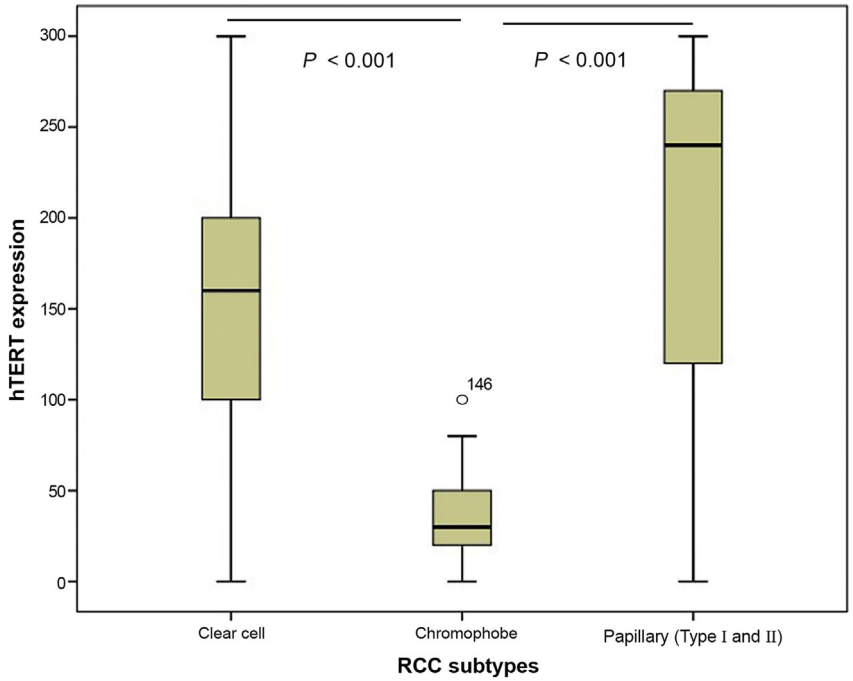

Fig. 2 Analysis of hTERT expression level in different renal cell carcinoma (RCC) subtypes including clear cell, chromophobe, and papillary RCC (type I and II) using Mann–Whitney *U* test. Based on the standard definitions, each box-plot shows the median (bold line), interquartile line (box), and outlier observation (circle). The results of Mann–Whitney *U* test showed statistically significant differences in the levels of hTERT expression between the ccRCC and chromophobe RCC ($p < 0.001$) and also type I and II papillary RCC and chromophobe RCC ($p < 0.001$).

Table 3 The association between human telomerase reverse transcriptase expression and clinicopathological parameters in three subtypes of RCC

RCC subtypes	Clear cell RCC (cut off = 160) *n* (%)		*p* value	Papillary RCC (Type I) (cut off = 140) *n* (%)		*p* value	Papillary RCC (Type II) (cut off = 240) *n* (%)		*p* value	Chromophobe RCC (cut off = 30) *n* (%)		*p* value
H-score	Low (≤160)	High (>160)		Low (≤140)	High (>140)		Low (≤240)	High (>240)		Low (≤30)	High (>30)	
	68 (60.2)	45 (39.8)		7 (58.3)	5 (41.7)		12 (60.0)	8 (40.0)		17 (54.8)	14 (45.2)	
Age, years	≤56	≤56	0.846	≤60	≤60	0.276	≤55	≤55	0.199	≤49	≤49	0.337
	>56	>56		>60	>60		>55	>55		>49	>49	
	33 (48.5)	21 (46.7)		5 (71.4)	2 (40.0)		4 (33.3)	5 (62.5)		8 (47.1)	9 (64.3)	
	35 (51.5)	24 (53.3)		2 (28.6)	3 (60.0)		8 (66.7)	3 (37.5)		9 (52.9)	5 (35.7)	
Gender												
Male	52 (76.5)	27 (60.0)	0.062	7 (100.0)	5 (100.0)	–[a]	9 (75.0)	5 (62.6)	0.550	9 (52.9)	9 (64.3)	0.524
Female	16 (23.5)	18 (40.0)		0 (0.0)	0 (0.0)		3 (25.0)	3 (37.5)		8 (47.1)	5 (35.7)	
Tumour size, cm												
0–4	20 (29.4)	5 (11.1)	**0.050**	2 (28.6)	0 (0.0)	0.330	3 (25.0)	1 (12.5)	0.298	1 (5.9)	2 (14.3)	0.131
4.1–7	23 (33.8)	17 (37.8)		3 (42.9)	1 (20.0)		2 (16.7)	4 (50.0)		6 (35.3)	7 (50.0)	
7.1–10	17 (25.0)	11 (24.4)		1 (14.3)	2 (40.0)		5 (41.7)	1 (12.5)		1 (5.9)	3 (21.4)	
>10.1	8 (11.8)	12 (26.7)		1 (14.3)	2 (40.0)		2 (16.7)	2 (25.0)		9 (52.9)	2 (14.3)	
Nucleolar grade												
I	0 (0.0)	0 (0.0)	**0.028**	0 (0.0)	0 (0.0)	0.310	0 (0.0)	0 (0.0)	0.494	0 (0.0)	0 (0.0)	–
II	51 (75.0)	27 (60.0)		6 (85.7)	3 (60.0)		3 (25.0)	1 (12.5)		0 (0.0)	0 (0.0)	
III	16 (23.5)	12 (26.7)		1 (14.3)	2 (40.0)		9 (75.0)	7 (87.5)		0 (0.0)	0 (0.0)	
IV	1 (1.5)	6 (13.3)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Primary tumour (PT) stage												
pT1	27 (39.7)	6 (13.3)	**<0.001**	5 (71.4)	0 (0.0)	**<0.001**	3 (25.0)	0 (0.0)	**0.050**	1 (5.9)	3 (21.4)	0.356
pT2	6 (8.8)	1 (2.2)		2 (28.6)	1 (20.0)		2 (16.7)	0 (0.0)		1 (5.9)	1 (7.1)	
pT3	35 (51.5)	30 (66.7)		0 (0.0)	4 (80.0)		4 (33.3)	5 (62.5)		15 (88.2)	9 (64.3)	
pT4	0 (0.0)	8 (17.8)		0 (0.0)	0 (0.0)		3 (25.0)	3 (37.5)		0 (0.0)	1 (7.1)	
Microvascular invasion (MVI)												
Present	6 (8.8)	13 (28.9)	**0.005**	0 (0.0)	0 (0.0)	–	2 (16.7)	2 (25.0)	0.648	6 (35.3)	4 (28.6)	0.690
Absent	62 (91.2)	32 (71.7)		7 (100.0)	5 (100.0)		10 (83.3)	6 (75.0)		11 (64.7)	10 (71.4)	
Lymph node invasion (LNI)												
Involved	2 (2.9)	6 (13.3)	**0.020**	0 (0.0)	0 (0.0)	0.217	2 (16.7)	2 (25.0)	0.648	0 (0.0)	0 (0.0)	–
None	66 (97.1)	37 (82.2)		7 (100.0)	4 (80.0)		10 (83.3)	6 (75.0)		17 (100)	14 (100)	
Not identified	0 (0.0)	2 (4.4)		0 (0.0)	1 (20.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Renal vein invasion												
Present	4 (5.9)	4 (8.9)	0.542	0 (0.0)	0 (0.0)	–	1 (8.3)	0 (0.0)	0.402	0 (0.0)	0 (0.0)	–
Absent	64 (94.1)	41 (91.1)		7 (100.0)	5 (100.0)		11 (9.1)	8 (100.0)		17 (100)	14 (100)	
Tumour necrosis												
Present	22 (32.4)	18 (40.0)	0.405	4 (57.1)	4 (80.0)	0.408	10 (83.3)	6 (75.0)	0.648	5 (29.4)	5 (35.7)	0.632
Absent	46 (67.6)	27 (60.0)		3 (42.9)	1 (20.0)		2 (16.7)	2 (25.0)		11 (64.7)	9 (64.3)	
Not identified	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		1 (5.9)	0 (0.0)	
Renal sinus fat invasion												
Present	28 (41.2)	32 (71.1)	**0.002**	0 (0.0)	2 (40.0)	0.067	8 (66.7)	7 (87.5)	0.292	15 (88.2)	7 (50.0)	0.053
Absent	40 (58.8)	13 (28.9)		7 (100.0)	3 (60.0)		4 (3.33)	1 (12.5)		2 (11.8)	7 (50.0)	
Renal pelvis invasion												
Present	1 (1.5)	6 (13.3)	**0.010**	0 (0.0)	0 (0.0)	–	2 (16.7)	3 (37.5)	0.292	0 (0.0)	0 (0.0)	–
Absent	67 (98.5)	39 (86.7)		7 (100.0)	5 (100.0)		10 (83.3)	5 (62.5)		17 (100)	14 (100)	
Perirenal fat invasion												
Present	8 (11.8)	11 (24.4)	0.078	0 (0.0)	1 (20.0)	0.217	2 (16.7)	2 (25.0)	0.648	3 (17.6)	3 (21.4)	0.791
Absent	60 (88.2)	34 (75.6)		7 (100.0)	4 (80.0)		10 (83.3)	6 (75.0)		14 (82.4)	11 (78.6)	
Gerota's fascia invasion												
Present	0 (0.0)	4 (8.9)	**0.012**	0 (0.0)	0 (0.0)	–	0 (0.0)	0 (0.0)	–	0 (0.0)	0 (0.0)	–
Absent	68 (100)	41 (91.1)		7 (100.0)	5 (100.0)		12 (100.0)	8 (100.0)		17 (100)	14 (100)	
Distant metastasis												
Present	12 (17.6)	16 (35.6)	**0.031**	0 (0.0)	0 (0.0)	–	1 (8.3)	4 (50.0)	**0.035**	1 (5.9)	3 (21.4)	0.199
Absent	56 (82.4)	29 (64.4)		7 (100.0)	5 (100.0)		11 (91.7)	4 (50.0)		16 (94.1)	11 (78.6)	

p value, Pearson's χ^2 test. Values in bold are statistically significant.
H-score, histological score; RCC, renal cell carcinoma.
[a] No statistics are computed because the parameter is constant.

Associations between hTERT protein expression and clinicopathological characteristics in RCC subtypes

Pearson's χ^2 test showed a significant association between hTERT expression and tumour stage ($p < 0.001$) as well as nucleolar grade ($p = 0.028$) in the ccRCC subtype. In addition, there was a significant association between higher levels of hTERT expression and tumour size ($p = 0.050$), MVI ($p = 0.005$), lymph node invasion (LNI) ($p = 0.020$), renal pelvis involvement ($p = 0.010$), renal sinus fat involvement ($p = 0.002$), Gerota's fascia invasion ($p = 0.012$), and distant metastasis ($p = 0.031$) (Table 3).

Kruskal–Wallis test indicated a statistically significant difference ($p < 0.001$) between median hTERT expression and various tumour stages (I–IV). The median expression level of hTERT was 100 in stage I, 71 in stage II, 178 in stage III, and 238 in stage IV. Moreover, Mann–Whitney *U* test

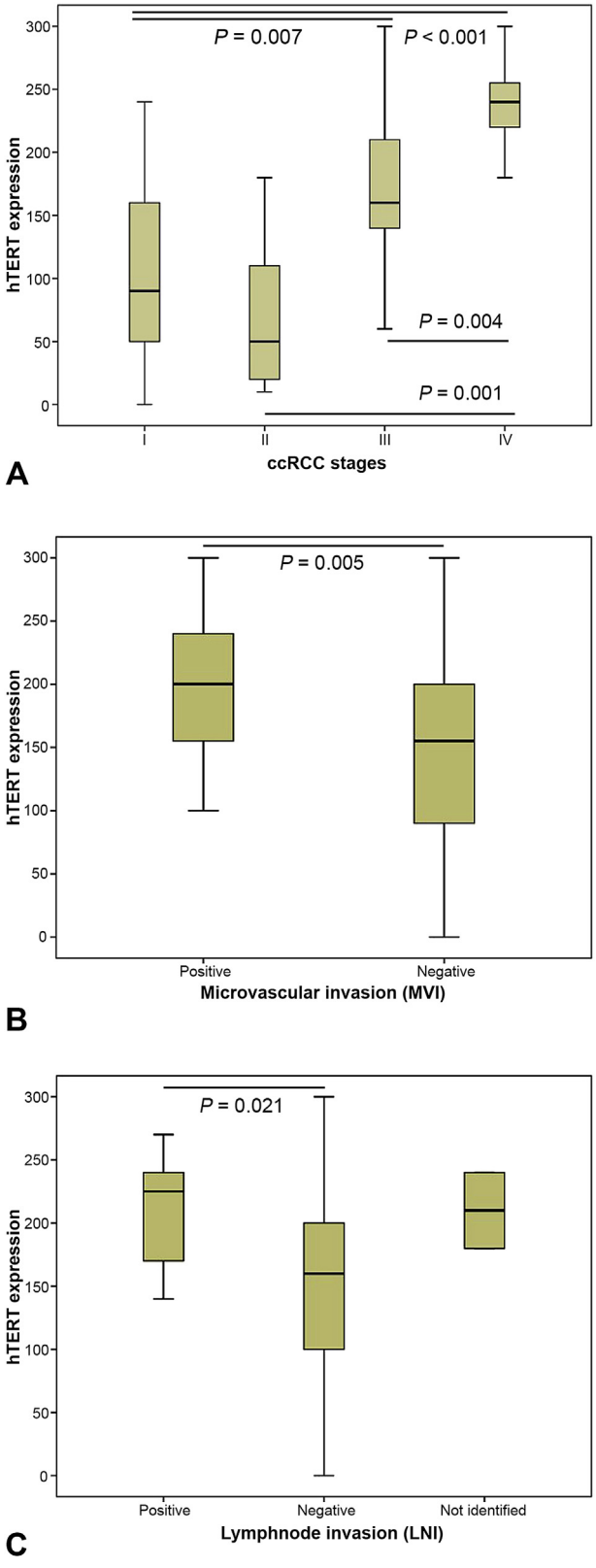

Fig. 3 (A) Box plot analysis of hTERT expression levels in stage I to IV, (B) microvascular invasion (MVI), (C) and lymph node invasion (LNI) in ccRCC, using Mann–Whitney *U* test. Based on the standard definitions, each box-plot shows the median (bold line) and interquartile lines (box). The result of Mann–Whitney *U* test showed that there was a statistically significant association for median of hTERT expression between stages I and IV ($p < 0.001$), stages I and III ($p = 0.007$), stages II and IV ($p = 0.001$) and stages III and IV ($p = 0.004$), and also median expression of hTERT and MVI ($p = 0.005$), as well as LNI ($p = 0.021$).

showed highly significant difference in the median of hTERT expression between stages I and IV ($p < 0.001$) (Fig. 3A).

The Kruskal–Wallis and Mann–Whitney *U* tests also showed statistically significant differences between median of hTERT expression and MVI ($p = 0.005$) as well as LNI ($p = 0.030$) (Fig. 3B,C).

Bivariate analysis showed a significant direct correlation between hTERT expression and advanced in tumour stage ($p < 0.001$) as well as nucleolar grade ($p = 0.049$). In addition, statistically significant correlation was observed between hTERT expression and MVI ($p = 0.005$), renal pelvis involvement ($p = 0.010$), renal sinus fat involvement ($p = 0.002$), Gerota's fascia invasion ($p = 0.012$), tumour size ($p = 0.017$), and distant metastasis ($p = 0.031$).

In type I and II papillary RCC, there was significant association between hTERT expression and tumour stage ($p = 0.010$, $p = 0.050$, respectively) and distant metastasis ($p = 0.035$) (Table 3). In chromophobe RCC, we did not find any association between hTERT expression and clinicopathological features (Table 3).

Prognostic value of hTERT expression for clinical outcome in RCC subtypes

Of the 176 RCC samples that were included in the present study, 134 (76.1%) patients had no history of metastasis and cancer-related death but 42 (23.9%) patients were positive for these parameters. Metastasis occurred in 37 (21.0%) patients. During follow-up time, cancer-related death occurred in 25 patients (14.2%). The mean duration of follow-up time was 43 months (SD = 19.6), median was 42.0 months (32, 58), and range was 1–78 months. Table 4 shows the main characteristics of patients enrolled for survival analysis according to RCC subtypes.

Kaplan–Meier survival analysis on ccRCC patients showed a significant difference between DSS among the high and low hTERT expressing patient groups (log rank test, $p = 0.012$). The mean DSS for patients with high and low hTERT expression was 58 (SD = 4.5) and 68 (SD = 2.3) months, respectively (Fig. 4A). The 5-year DSS for high and low hTERT expressing patients was 72% and 91%, respectively ($p = 0.017$).

Significant risk factors affecting DSS in univariate analysis included hTERT expression ($p = 0.019$), nucleolar grade ($p = 0.003$), tumour stage ($p < 0.001$), and tumour size ($p < 0.001$). Other clinicopathological parameters did not significantly affect DSS in ccRCC patients. In multivariate analysis, tumour stage ($p = 0.044$) and tumour size ($p = 0.014$) were significantly related to DSS in ccRCC patients (Table 5).

In type I papillary RCC there was no cancer-related death, therefore it was not possible to draw the Kaplan–Meier survival curve; however, four patients with type II papillary RCC were lost to renal cancer (Table 4). The results showed that the patients with high expression of hTERT had shorter disease-specific survival compared to patients with low hTERT expression, but log rank test was not significant ($p = 0.117$) (Fig. 4B). In chromophobe RCC patients, Kaplan–Meier survival analysis showed that high levels of hTERT expression were not significantly related to DSS (Fig. 4C). In addition, results of univariate and multivariate analyses demonstrated that clinicopathological variables

Table 4 The main characteristics of patients enrolled for survival analysis according to RCC subtypes

Feature	RCC subtypes			
	Clear cell RCC	Papillary RCC		Chromophobe RCC
		Type I	Type II	
Number of patients, *n* (%)	113	12	20	31
Mean duration of follow-up time, months (SD)	40 (20.1)	55 (15.1)	48 (21.7)	47 (15.4)
Median duration of follow-up time, months (Q1, Q3)	42 (27, 55)	55 (39, 72)	42 (37, 66)	39 (36, 61)
Cancer-related death, *n* (%)	18 (15.9)	0 (0.0)	4 (20.0)	3 (9.7)
Metastasis history during follow-up, *n* (%)	28 (24.8)	0 (0.0)	5 (25.0)	4 (12.9)
Alive patients without any complication, *n* (%)	81 (71.7)	12 (100.0)	15 (75.0)	26 (83.9)

RCC, renal cell carcinoma.

were not significant factors affecting DSS in patients with papillary (type I and II) and chromophobe RCC.

DISCUSSION

Telomerase is an attractive and ideal target for therapy due to overexpression in the majority of malignancies and low or non-expression in most somatic cells.[9,10] Several studies have previously investigated levels of telomerase activity and hTERT mRNA expression in RCC,[12–14,27] whereas we could not find any study addressing hTERT protein expression in RCC patients.

For the first time, in the present study we investigated the expression levels of hTERT protein in a well-characterised series of 176 RCC tissue samples from patients treated with radical nephrectomy. There were highly significant associations between the level of hTERT expression and RCC subtype. Moreover, a significant difference was observed in the levels of hTERT expression between ccRCC and chromophobe samples and also type I and II papillary and chromophobe RCC. These findings demonstrate that hTERT has different expression patterns among various subtypes of RCC, implying molecular mechanisms which lead to cellular immortality may differ according to tumour subtype.[28]

In this study, we showed that hTERT protein expression is associated with more advanced tumour stage and higher nucleolar grade in ccRCC. The tumour stage and nucleolar grade are the most reliable prognostic factors in RCC and tumours with a high nucleolar grade have a more aggressive phenotype.[29] Importantly, we observed that the median expression of hTERT was higher in more advanced tumour stages (stage III and stage IV) when compared to stages I and II, which depicts the association of hTERT protein expression with aggressiveness of ccRCC. Also, tumour stage and nucleolar grade were significantly correlated with poor prognosis; however, tumour stage was found to be an independent prognostic factor in multivariate analysis. The current study demonstrated a trend towards the association of increased hTERT protein expression and stage. Our result is in agreement with a study performed by Mekhail *et al.*[14] who showed that relationship between telomerase activity and tumour stage. In addition, significant association was found between the level of hTERT expression and distant metastasis. This observation is also consistent with a previous report based on the measurement of serum hTERT enzyme activity showing that its levels are associated with more advanced tumour stage and distant metastasis.[30] Moreover, the presence of a statistically significant association between expression levels of hTERT and tumour size, renal pelvis and renal sinus fat involvement, and Gerota's fascia invasion showed that hTERT protein expression is related to the degree of malignancy and more advanced disease in ccRCC. Evidence shows that hTERT plays a role in tumour invasion and metastasis by promoting epithelial mesenchymal transition (EMT). Also, hTERT can activate the WNT/β-catenin signalling pathway; therefore, it may contribute to cancer stem cell (CSC) maintenance.[31] CSCs, a subpopulation among the tumour cells, have the ability to self-renew and have high tumour-initiating potential; thus, they are able to drive cancer maintenance, progression, and metastasis.[32] Moreover, it is known that EMT generates cancer cells with stem cell-like characteristics; stem-like cells express markers associated with EMT; and the diversity and abundance of CSCs in solid tumours allow cells the ability to undergo EMT and cancer progression.[33] Castelo-Branco *et al.* showed that CSCs have significantly higher levels of hTERT expression and extremely short telomeres compared with normal tissue stem cells.[34] CD133 as a CSC marker was more highly expressed in hTERT-immortalised cells than in primary prostate cells.[35]

In our study, tumour size was found to be an independent prognostic indicator in multivariate analysis, in contrast with some previous studies which could not find any significant association between telomerase activity and tumour size.[13,36] Investigations have shown that tumour size is an important clinical and pathological feature for patients with RCC and is significantly associated with risk of metastasis.[37,38]

RCC is clinically recognised as a highly vascularised tumour, and one of the features of ccRCC is the presence of branching, thin-walled vessels in between the tumour cells.[24] Interestingly, we observed higher level of hTERT protein in this tumour subtype associated with higher MVI. Lang *et al.*[39] showed that MVI is related to cancer progression and survival in RCC and has more effect on prognosis compared to the macroscopic renal vein or vena cava invasion after treatment by radical nephrectomy. LNI is also one of the other important prognostic factors in RCC. A previous study showed that LNI was independently associated with cancer specific survival after surgical resection and provides the strongest predictor of prognosis in patients without MVI.[40]

Our results are in agreement with the previous studies which concluded that hTERT protein has an important role in advanced malignancy in some cancer types including breast cancer, gastric cancer, colorectal cancer, meningiomas, and spinal chordoma.[41–45]

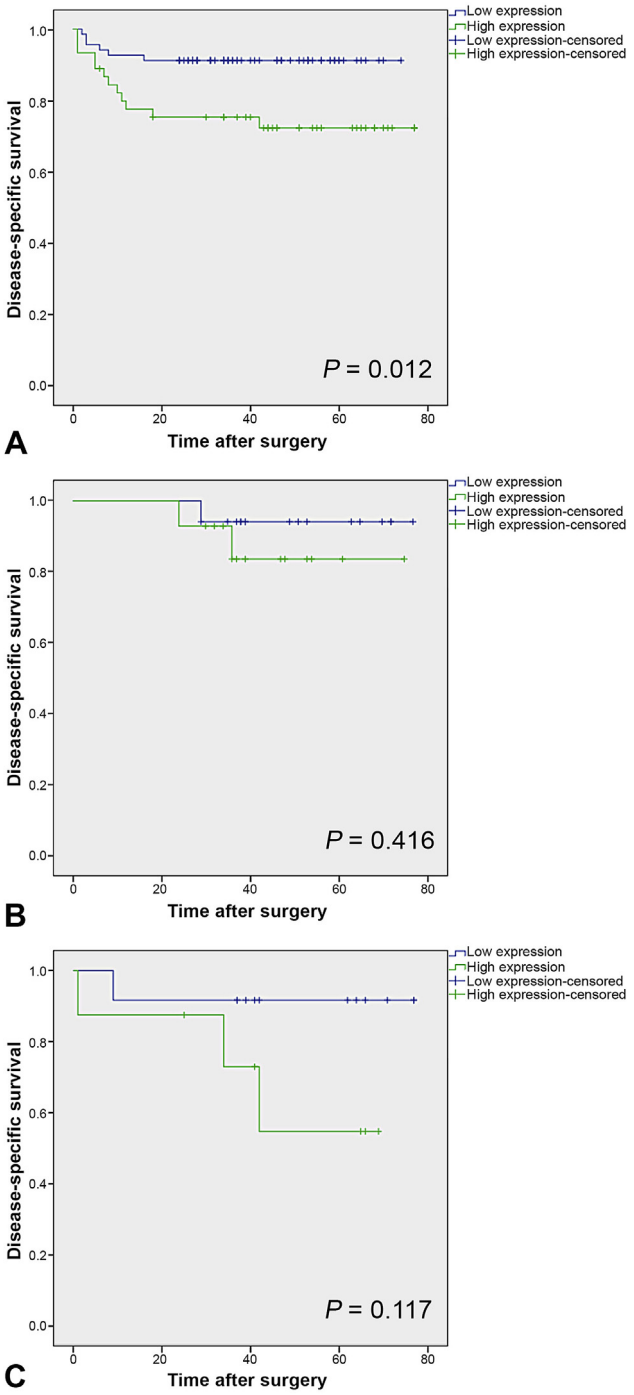

Fig. 4 Kaplan–Meier curves for disease-specific survival (DSS) based on hTERT protein expression level in ccRCC. (A) In ccRCC patients, higher level of hTERT protein expression was associated with shorter disease-specific survival compared to the tumours with low expression of this protein ($p = 0.012$). (B,C) In type II papillary and chromophobe RCC patients, Kaplan–Meier survival analysis showed that high levels of hTERT expression are not significantly related to DSS ($p = 0.416$, $p = 0.117$, respectively).

For the first time, we found that tumours with higher expression of hTERT tend to have a worse prognosis compared to those with low expression. In addition, ccRCC patients who expressed a higher level of hTERT had a shorter 5-year disease-specific survival compared with those with low expression. To our knowledge, this is the first study showing that hTERT protein expression is a prognostic marker in ccRCC, although without an independent statistical significance in multivariate analysis. In our study, the number of cancer-related deaths or events was low at 18 (15.9%); therefore, more prolonged follow-up time seems to be important because by extending the follow-up time, the prognostic value of hTERT expression may be more accurately estimated. Previous studies which focused on hTERT mRNA expression in RCC samples concluded that telomerase activity was not related to prognosis.[12–14] A possible explanation for this discrepancy is post-transcriptional modification of hTERT mRNA.

We also examined hTERT protein expression patterns in 12 type I, 20 type II papillary and 31 chromophobe RCC samples which are less frequent variants of RCC. We showed that in papillary RCC (type I and II), there was significant association between increased hTERT protein expression and more advanced disease, especially in type II papillary RCC. Although there was no statistically significant association between hTERT protein expression and survival, the patients with high expression of hTERT had shorter disease-specific survival compared to patients with low hTERT expression in type II papillary RCC. This result might be due to the low number of cases and events; therefore, a larger sample size and a lengthier follow up may be required to clearly address this issue. Moreover, in chromophobe RCC, no significant association was found between hTERT expression and tumour aggressiveness. This finding is in agreement with a previous study that telomerase was found only infrequently in chromophobe RCC.[46] ccRCC is known to be more aggressive and associated with poorer prognosis than papillary and chromophobe RCC subtypes.[47] Almost all ccRCCs have deletions in the short arm of chromosome 3, resulting in mutations of the von Hippel–Lindau (VHL) gene which are frequent in ccRCC and are not observed in any other subtypes.[48] Evidence shows that mutations of VHL tumour suppressor gene lead to loss of function of the VHL tumour suppressor protein and consequent activation of the hypoxia pathway via the hypoxia-inducible factors,[49] like transcription factor HIF1α which plays an important role in development of ccRCC and upregulates a series of hypoxia-responsive genes, including VEGF which has a pivotal role in ccRCC tumourigenesis.[50] Also, it has been previously reported that transcription factor HIF1α induces hTERT expression and the level of hTERT is increased under hypoxic conditions.[51]

A limitation of the present study was to carry out the test only on TMAs of RCC subtypes. Although TMA technology is a very attractive method for high throughput analysis of hundreds of tissues simultaneously,[52] it may have limitations. A disadvantage of TMA technology compared to whole tissue sections is that the TMA cores may not be representative for the whole tumour, particularly in the case of heterogeneous tumours and also heterogeneity in the expression of molecular markers in tumour tissue.[53] In the present study, three cores from the most representative areas of tumours were selected and scored individually to overcome the heterogeneity of hTERT protein expression. No variability was seen in three cores of each sample in the IHC staining,

Table 5 Univariate and multivariate Cox regression analyses of potential prognostic factors for disease-specific survival in patients with clear cell RCC

Covariate	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
H-score				
High versus Low	3.246 (1.217–8.655)	**0.019**	1.040 (0.290–3.736)	0.952
Tumour size (cm)	2.657 (1.567–4.503)	**<0.001**	2.279 (1.185–4.382)	**0.014**
Nucleolar grade		**0.003**		0.585
III versus II	4.929 (1.751–13.880)	**0.003**	1.875 (0.564–6.229)	0.305
IV versus II	6.712 (1.674–26.919)	**0.007**	1.735 (0.357–8.430)	0.494
TNM stage		**<0.001**		**0.044**
pT2 versus pT1	5.424 (0.339–86.873)	0.232	1.156 (0.056–23.841)	0.925
pT3 versus pT1	4.968 (0.629–39.226)	0.128	1.524 (0.147–15.822)	0.724
pT4 versus pT1	47.149 (5.732–387.856)	**<0.001**	9.572 (1.042–123.459)	**0.050**

CI, confidence interval; HR, hazard ratio; H-score, histological score; RCC, renal cell carcinoma.

however our findings should be confirmed on a number of whole sections from each of the three RCC subtypes in a future study.

CONCLUSION

From these findings we are able to conclude that hTERT protein expression may be a novel prognostic indicator of worse outcome in tumour biopsies of patients with ccRCC, if follow up time is more prolonged. In addition, increased hTERT protein expression may indicate more aggressive tumour behaviour and more advanced disease in ccRCC cases. Thus, evaluation of hTERT protein expression can be useful for predicting tumour invasiveness in ccRCC patients. Further studies are required using full sections of the three RCC subtypes to confirm our findings.

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