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Diagnostic and prognostic value of the detection of hTERT mRNA in renal tumors

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Abstract

Introduction: Elevated mRNA expression of human telomerase reverse transcriptase (hTERT mRNA) is common in many types of tumors, participating in tumor growth and progression. Such expression has not been sufficiently examined in renal cancer. The goal of the present study was to quantify it and analyze its possible clinical value in the management of this pathology.

Patients and methods: The study included 111 patients who underwent surgery for renal cell carcinoma (RCC) between 2015 and 2017. Tumor samples were taken from all patients and, in 94 of them, healthy renal tissue adjacent to the tumor was also sampled. The 2 types of tissue were histologically confirmed, after which mRNA was extracted. Using real-time quantitative PCR, the expression of hTERT and glyceraldehyde-3-phosphate dehydrogenase (as endogenous control) were indirectly quantified using the crossing point (CP), which is inversely correlated with the number of sample replicates yielding positive results. These values were correlated with patient socio-demographic variables and clinical-pathological factors of the RCC.

Results: The majority of patients were males, with an average age of 60.5 years (SD: 14.02). Most tumors (69.4%) were clear cell carcinomas. The most frequent stages were pT2 or lower (73%), while 5% were pN1 and 12% pM1. The majority of tumors (58%) were Fuhrman grades 1 or 2 (low grade). All samples of tumor and nontumor tissue expressed glyceraldehyde-3-phosphate dehydrogenase mRNA, with the CP in the tumor sample significantly lower than in the nontumor tissue ($P < 0.001$). The expression of hTERT mRNA was detected in 68% of tumor tissues and significantly correlated with histopathology: 100% in sarcomatoid RCC and 77.9% in clear cell carcinomas ($P < 0.0001$). The CP was lower in pN1 ($P = 0.018$), pM1 ($P = 0.046$), and TNM IV stages ($P = 0{,}041$). A greater number of hTERT mRNA replicas were detected in M1 patients ($P = 0.0005$) and TNM IV stage ($P = 0.017$). There was no correlation of hTERT mRNA expression with Fuhrman grade.

Conclusions: The quantitation of hTERT mRNA expression in RCC might be useful as a complementary diagnostic tool as well as for assessing aggressiveness of the tumor.

Keywords: Renal cell carcinoma; hTERT mRNA; Diagnosis; Prognosis

Abbreviations: CP, crossing point; hTERT mRNA, human telomerase reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene; RCC, renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; BMI, body mass index

1. Introduction

Renal cell carcinoma (RCC) is the most common solid lesion in the kidney and accounts for approximately 90% of all malignant renal tumors. There are different types of RCC, with specific histopathological and genetic characteristics [1]; the most frequent is clear cell carcinoma (ccRCC; 80%–90%), followed by papillary and chromophobe (15% and 5%, respectively). RCC shows important heterogeneity, derived from its different histological forms and its genetic

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and molecular variants, making it a difficult entity to manage and approach globally.

The diagnostic and prognostic markers in this type of cancer are based only on the clinical-pathological history (TNM, Fuhrman grade, histological type, and molecular factors) and radiological examinations, due to the lack of blood and urine markers. The search for specific markers is essential for improving the management of RCC patients, from diagnosis through to treatment and eventually follow-up [2].

Telomerase is an enzyme with an important role in the repair and lengthening of telomeres at the end of eukaryotic chromosomes, preventing cellular senescence and programmed death, and is over-expressed in a variety of tumors (such as laryngeal) [3]. Telomerase is a ribonucleoprotein that contains an internal RNA template (hTR) and a catalytic protein with reverse transcriptase action (hTERT). The expression of telomerase is practically undetectable in nontumor tissues, except for in certain cells such as stem cells, lymphocytes, germ cells, oral mucosa, or endometrium in the proliferative phase of the menstrual cycle. The expression of hTERT mRNA has been determined in 80% to 90% of human tumors and its activation has been demonstrated in premalignant lesions [4]. Due to its strong link to tumor stage, hTERT mRNA has been proposed as a marker for diagnosing and monitoring various types of cancers, such as colorectal [5], breast [6], and prostate cancer [7].

With respect to renal cancer, expression of hTERT mRNA has been detected in tumor tissues [8,9], although the clinical value of such expression has not been examined. The aim of the present study was to quantify hTERT mRNA in RCC and to determine its possible diagnostic and prognostic value in this pathology.

2. Patients and methods

We recruited 111 patients who underwent surgery for RCC between March 2015 and February 2017 in the Urology Service of the University General Hospital of Albacete (Spain). Tumor samples were removed from all patients and, in 94 of them, samples of macroscopically healthy renal tissue adjacent to the tumor were also taken. Histological analysis was performed on all samples. The study was approved by the local research ethics committee, and all samples were obtained following patients´ written informed consent.

All patients with RCC diagnose during the recruitment period were included in the study. Oncocytoma cases were also included, due to its possible association with other histological subtypes. Those cases with only oncocytoma subtype, other benign conditions, and urothelial tumors were excluded.

2.1. Extraction of RNA from tumor samples and paired adjacent nontumor tissues

RNA was extracted from tissues using a commercial kit (All Prep DNA/RNA Mini; Qiagen, Hilden, Germany). After extraction, the RNA samples were incubated with DNase I (RNase-free DNase set, Qiagen) to eliminate any contamination by DNA.

2.2. Amplification and quantitation of complementary DNA (cDNA)

Samples of complementary DNA (cDNA) were analyzed by real-time PCR with fluorescent hybridization probes for amplification of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*-specific and *hTERT*-specific sequences, respectively. In the case of GAPDH, we amplified a 226-bp sequence with forward and reverse primers 5′-gaaggtgaaggtcggagtc3′ and 5′-gaagatggtgatgggatttc-3′, respectively. We used a TaqMan probe for detection and quantitation of GAPDH cDNA sequences. The probe was labeled at the 5′ end with 6-carboxyfluorescein (FAM) dye and at the 3′ end with tetramethylrhodamine (TAMRA) to yield the following oligonucleotide: 5′-FAM-caagcttcccgttctcagcc-TAMRA. In the case of hTERT, we amplified a 95-bp sequence with the forward and reverse primers 5′-tgacacctcacctcacccac-3′ and 5′-cactgtcttccgcaagttcac-3′, respectively. The hTERT-specific TaqMan probe yielded the following sequence: 50-FAM-accctggtccgagg tgtgtccctgag-TAMRA. The probes were manufactured by TIB MOBIOL (Berlin, Germany). For PCR, we used an LC Fast Start DNA MasterPLUS Hyb Probes kit that included FastStart Taq DNA polymerase, reaction buffer, magnesium chloride, and deoxynucleotide triphosphate mixture (Roche Diagnostics GmbH). The reaction mixture contained 2 ll of cDNA solution, 0.5 lM of each primer, 0.2 lM of probe, and 3 mM $MgCl_2$, for a total volume of 20 ll. In all amplification experiments, DNA from RCC and water were included as positive and negative controls, respectively.

We performed all PCRs with the LightCycler System (Roche Diagnostics), using LightCycler software version 4.0. Samples were placed in capillary tubes and subjected to initial denaturation by incubation at 95˚C for 5 minutes. Then, amplification was allowed to proceed for the indicated number of cycles of denaturation and annealing/extension, namely 45 cycles for amplification of *GAPDH* cDNA and 47 cycles for *hTERT* cDNA. The first step in the amplification was incubation for 15 seconds at 95˚C. The annealing/extension incubation was allowed to proceed for 30 seconds at 59˚C in the case of GAPDH cDNA and for 30 seconds at 65˚C for hTERT cDNA. During the second incubation (annealing step), fluorescence was monitored at 530 nm (F1 channel in the LightCycler system). Each reaction was performed in triplicate.

The results of sample analysis in the LightCycler were expressed in terms of crossing point (CP) value, defined as the cycle number at which fluorescence is first detected during exponential increase of replicas (Fig. 1). The CP values are inversely proportional to the expression levels of hTERT mRNA.

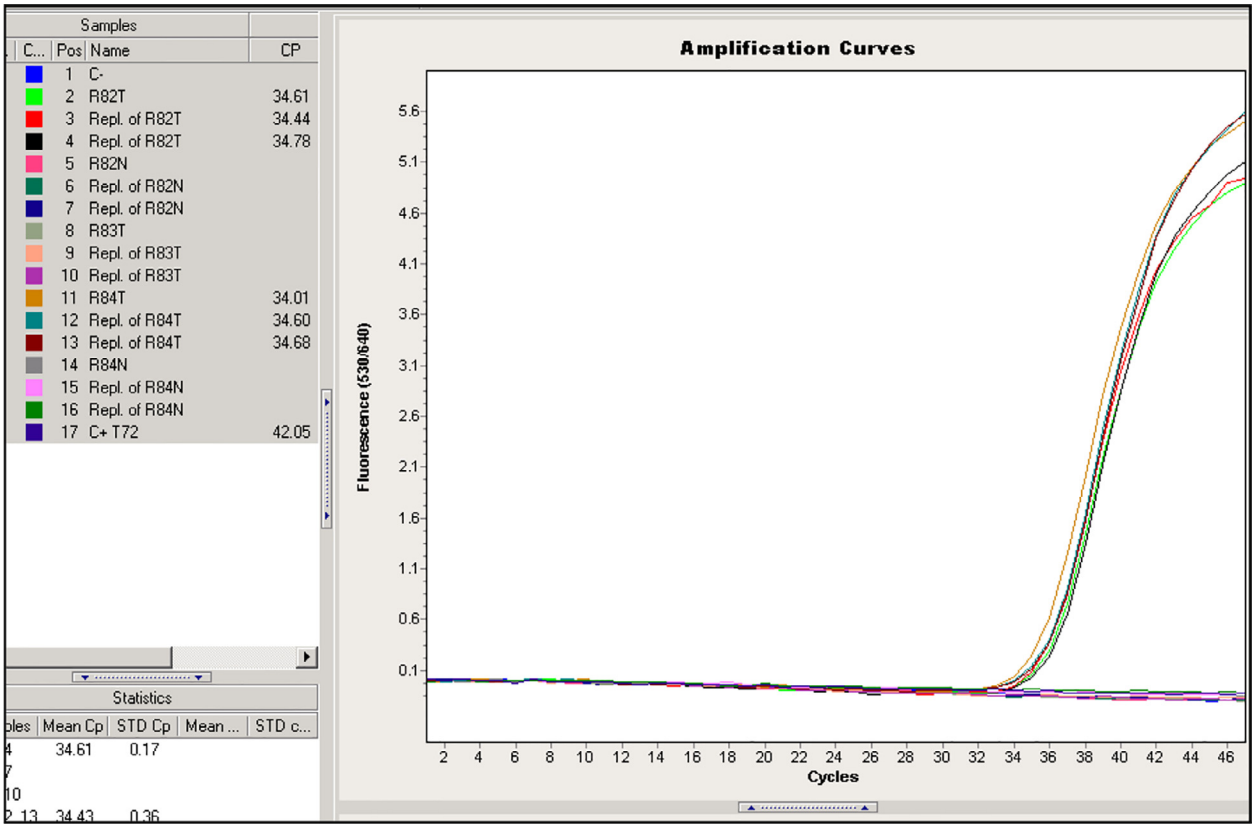

Fig. 1. Graph of hTERT mRNA crossing points.

Data were obtained from patient medical records regarding age, sex, clinical diagnosis, clinical history related to the development of RCC, and anatomopathological characteristics of the tumor: laterality, size, multiplicity, histopathological diagnosis, Fuhrman grade, and TNM stage [10]. The N stages were evaluated as N1 (lymph node involvement in lymphadenectomy) and N0 (no lymph node involvement in either lymphadenectomy specimen or imaging tests).

2.3. Statistical methodology

A descriptive, bivariate analysis of the study variables was carried out. Contingency and comparison of means tests were carried out, depending on the type of variable, to determine whether there were any significant differences. In those cases, in which the application criteria of the previous tests were not valid, their corresponding nonparametric tests were used. The analysis was performed with the statistical program SPSS.

3. Results

3.1. Sociodemographic and clinical-histopathological characteristics of patients

The average age of patients was 60.5 years. A 69.4% were male; age and sex were not significantly related. Clinical history revealed that 55.9% were smokers and 44.1% hypertensive. In addition, the average body mass index of the patients was 28.2. The predominant clinical presentation was incidental in 76.6% of patients, followed by hematuria in 9.9%. Radical nephrectomy was carried out in 79.2% of cases, with predominance of lumbotomy (35.1%), followed by laparoscopy (29.7%). Partial surgery was performed on 20.7%. Perioperative complications occurred in 7.2% (Table 1).

Regarding histopathology, 69.4% of cases were ccRCC, 11.7% papillary RCC, 9.9% chromophobe, 7.2% oncocytomas, and 1.8% were sarcomatoid carcinomas. Fifty-seven percent of cases were stage pT1, with predominance of pT1a (33%); 16% were stage pT2, and 27% were diagnosed in stages pT3 or pT4. Lymph node involvement was positive in 5.1% of patients. A 12.6% were male presented with metastatic disease, with no significant pattern in terms of location of metastases. Regarding the degree of Fuhrman differentiation, 58% of cases presented grades 1 or 2. The most frequently encountered TNM stage was I (Table 1).

3.2. Detection of GAPDH mRNA in tumor tissue and in paired adjacent nontumor tissue

All samples of tumor tissue and paired adjacent nontumor tissue expressed GAPDH mRNA, with an average CP of 24.3 in tumor samples (confidence intervals [CI] 95%:

Table 1
Distribution of clinical-histopathological variables

Clinical-histopathological variables		*n* (%)
Sex	Men	80 (72.0)
	Women	31 (28.0)
Laterality	Right	56 (50.4)
	Left	55 (49.6)
Presenting symptoms	Incidental	89 (80.2)
	Hematuria	9 (8.1)
	Back Pain	4 (3.6)
	Pain + Hematuria	3 (2.7)
	Weight loss	2 (1.8)
	Other	4 (3.6)
Adyuvant treatment	Chemotherapy	7 (5.2)
	Radiotherapy	3 (1.7)
Body mass index	Normal	30 (26.7)
	Overweight	44 (40)
	Obesity	22 (20)
	Severe obesity	10 (9.2)
	Morbid obesity	5 (4.2)
Risk factors	HTA	64 (57.5)
	Smoking	65 (58.3)
	Neoplasm	4 (3.3)
	Renal insufficiency	4 (3.3)
Histology	Clear cell	77 (69.4)
	Papillar	13 (11.7)
	Chromophobe	11 (9.9)
	Sarcomatoid	2 (1.8)
	Oncocytomas	8 (7.2)
Fuhrman degree	1	7 (7.5)
	2	47 (50.5)
	3	27 (29)
	4	12 (12.9)
pT	T1	57 (57.0)
	T2	16 (16.0)
	T3	2 (22.0)
	T4	5 (5.0)
N	0	94 (94.9)
	1	5 (5.1)
pM	0	90 (87.3)
	1	13 (12.7)
TNM stage	I	39 (52.7)
	II	11 (14.9)
	III	11 (14.9)
	IV	13 (17.6)

23.6–25.01); this result is significantly lower compared to the CP of nontumor tissue (mean: 27.2 CI 95%: 26.4–28.0, $P < 0.001$).

3.3. Detection of hTERT mRNA in tumor tissue and in paired adjacent nontumor tissue

Sixty-eight percent of tumor tissue samples expressed hTERT mRNA; this occurred in only 33% of healthy tissue samples adjacent to the tumor ($P < 0.001$).

The expression of hTERT mRNA was detected in 100% of the sarcomatoid RCCs, in 77.9% of the ccRCCs and in 69.2% of the papillary RCCs. These differences in expression of hTERT mRNA according to histological subtypes were statistically significant ($P < 0.0001$). There were no other histopathological variables associated with the expression of hTERT mRNA. These data are shown in Table 2.

There were also significant differences in the CPs of hTERT mRNA according to the different histological subtypes ($P < 0.0001$). The mean CP of hTERT mRNA was 36.6 (CI 95%: 36.1–37.1), indicating that the CP for the sarcomatoid RCCs was significantly lower than for the rest of the histological subtypes (mean: 29.2, CI 95%: 22.1–36.2); this implies greater expression of hTERT mRNA in this subtype. Table 3 shows the different CPs according to the histopathological variables analyzed and demonstrates that the CPs of metastatic tumors ($P = 0.046$; Fig. 2) with lymph node involvement ($P = 0.048$; Fig. 2) in TNM stage IV ($P = 0.041$; Fig. 3), were significantly lower in relation to those of nonmetastatic tumors, cN0, and in lower TNM stages.

All of the sarcomatoid tumors showed 3 replicas for hTERT mRNA, while in 51.9% of the ccRCCs 2 or 3 replica were detected ($P = 0.002$). In 1 of the 8 oncocytomas (12.5%), a single replica was found.

Although 80% of the N1 cases expressed 3 replicas for hTERT mRNA, this figure did not differ significantly from that of patients with no evidence (clinical or pathological) of lymph node involvement.

According to expression or not of hTERT mRNA, there was no significant difference in the percentage of expression between pM0 and pM1 patients (Table 2). However, 85% of pM1 patients showed 3 replicas, while only 32% of M0 patients expressed such a result ($P = 0.005$).

Patients in stage TNM IV showed a higher percentage of number of replicas compared to the other TNM stages (71.5% had 3 replicas vs. an average of 32% in lower TNM stages $P = 0.017$; Fig. 4). Table 4 shows the distribution of hTERT mRNA replicas according to the different histopathological variables of the study.

4. Discussion

It has been reported that telomerase is activated in 80% to 90% of human carcinomas, but not in normal somatic cells; therefore, its detection is promising as a diagnostic marker for cancer [4,11]. With regard to renal cancer, Rohde et al. [12] evaluated the expression of hTERT mRNA in 35 cases of RCC, finding a positive result in 75% of samples. They also included 2 angiomyolipomas and 1 urinary tract tumor negative for telomerase activity, suggesting a relationship between telomerase and RCC. Furthermore, Pal et al. [11] found that after analyzing 96 cases of RCC, hTERT mRNA was significantly over-expressed in RCC tissues, whereas it remained undetected in healthy tissue samples.

In our series, we observed that 68% of tumor tissues were positive for the expression of hTERT mRNA; however, the same result occurred in 33% of healthy kidney tissue samples adjacent to the tumor. This finding is consistent

Table 2
hTERT mRNA expression according to histopathological variables

Histopathological variables		hTERT mRNA expression		*P*
		YES *n* (%)	NO *n* (%)	
Histology	Clear cell	60 (77.9)	17 (22.1)	<0.0001
	Papillar	9 (69.2)	4 (30.8)	
	Chromophobe	4 (36.4)	7 (63.6)	
	Sarcomatoid	2 (100)	0 (0.0)	
	Oncocytomas	1 (12.5)	7 (87.5)	
Fuhrman degree	1	4 (57.1)	3 (42.9)	NS
	2	33 (70.2)	14 (29.8)	
	3	22 (81.5)	5 (18.5)	
	4	10 (83.3)	2 (16.7)	
pT	1	39 (68.4)	18 (31.6)	NS
	2	12 (75.0)	4 (25.0)	
	3	19 (86.4)	3 (13.6)	
	4	4 (80.0)	1 (20.0)	
cN	0	70 (74.5)	24 (25.5)	NS
	1	4 (98.6)	1 (1.4)	
pM	0	63 (70.0)	27 (30.0)	NS
	1	12 (92.3)	1 (7.7)	
TNM stage	I	39 (67.2)	19 (32.8)	NS
	II	11 (73.3)	4 (26.7)	
	III	11 (73.3)	4 (26.7)	
	IV	13 (92.9)	1 (7.1)	

with that of a previous study in which the expression of hTERT mRNA was detected in both tumor tissue and healthy tissue [13]. The presence of hTERT mRNA in healthy tissue has been interpreted as an effect derived from the inflammatory reaction associated with the tumor growth process, mediated mainly by lymphoid cells [4].

Table 3
Distribution of hTERT mRNA crossing points according to histopathological variables

Histopathological variables		Mean	CI 95%	*P*
Histology	Clear cell	36.9	36.4–37.3	<0.001
	Papillar	36.0	34.4–37.6	
	Chromophobe	37.5	36.7–38.2	
	Sarcomatoid	29.2	22.1–36.2	
	Oncocytomas	38.3	-	
Fuhrman degree	1	37.5	37.0–38.1	NS
	2	36.9	36.4–37.5	
	3	36.7	35.7–37.8	
	4	35.7	36.9–37.4	
pT	1	36.9	36.2–37.6	NS
	2	36.5	35.6–37.4	
	3	36.5	35.4–37.6	
	4	34.1	29.2–39.5	
N	0	36.7	36.2–37.2	0.018
	1	33.9	29.3–38.5	
pM	0	36.9	36.4–37.4	0.046
	1	34.9	33.1–36.8	
TNM stage	I	36.9	36.2–37.6	0.041
	II	36.6	35.5–37.6	
	III	37.4	36.6–38.1	
	IV	35.1	33.3–36.8	

On the other hand, 79% of the ccRCCs in our study showed significant expression of hTERT mRNA, a much higher rate than for other histological types (with exception of 2 cases of RCC sarcomatoid) suggesting that hTERT mRNA could serve as a marker for histological classification of RCC. Moreover, in our series, the only oncocytoma out of 8 (12.5%) that expressed hTERT was of mixed histology (with a minor sarcomatoid component), further supporting hTERT's possible role in histopathological diagnosis of RCC. The expression of hTERT mRNA in RCC has been reported in the literature, but we have not found any published data linking hTERT mRNA expression rates to different histological subtypes of RCC, as we have done in this study.

In quantifying hTERT mRNA (according to CP values), we found that patients with lymph node involvement (N1) expressed higher levels of this potential marker than N0 patients. This finding may imply that the load of hTERT mRNA in N1 patient, would be greater and, therefore, would be detected earlier, giving this data a potential prognostic value when determining hTERT mRNA.

García Olmo et al. [3] found expression of hTERT mRNA in 72% of samples from patients with laryngeal cancer, with higher rates of expression in subjects with metastasis. This phenomenon was also observed in the renal cancer patients in our study, where 92% of metastatic patients expressed hTERT mRNA in tumor samples at diagnosis. Moreover, our results agree with those of Pal et al. [11] and Zhou et al. [14], who reported increased expression of hTERT mRNA associated with tumor invasiveness.

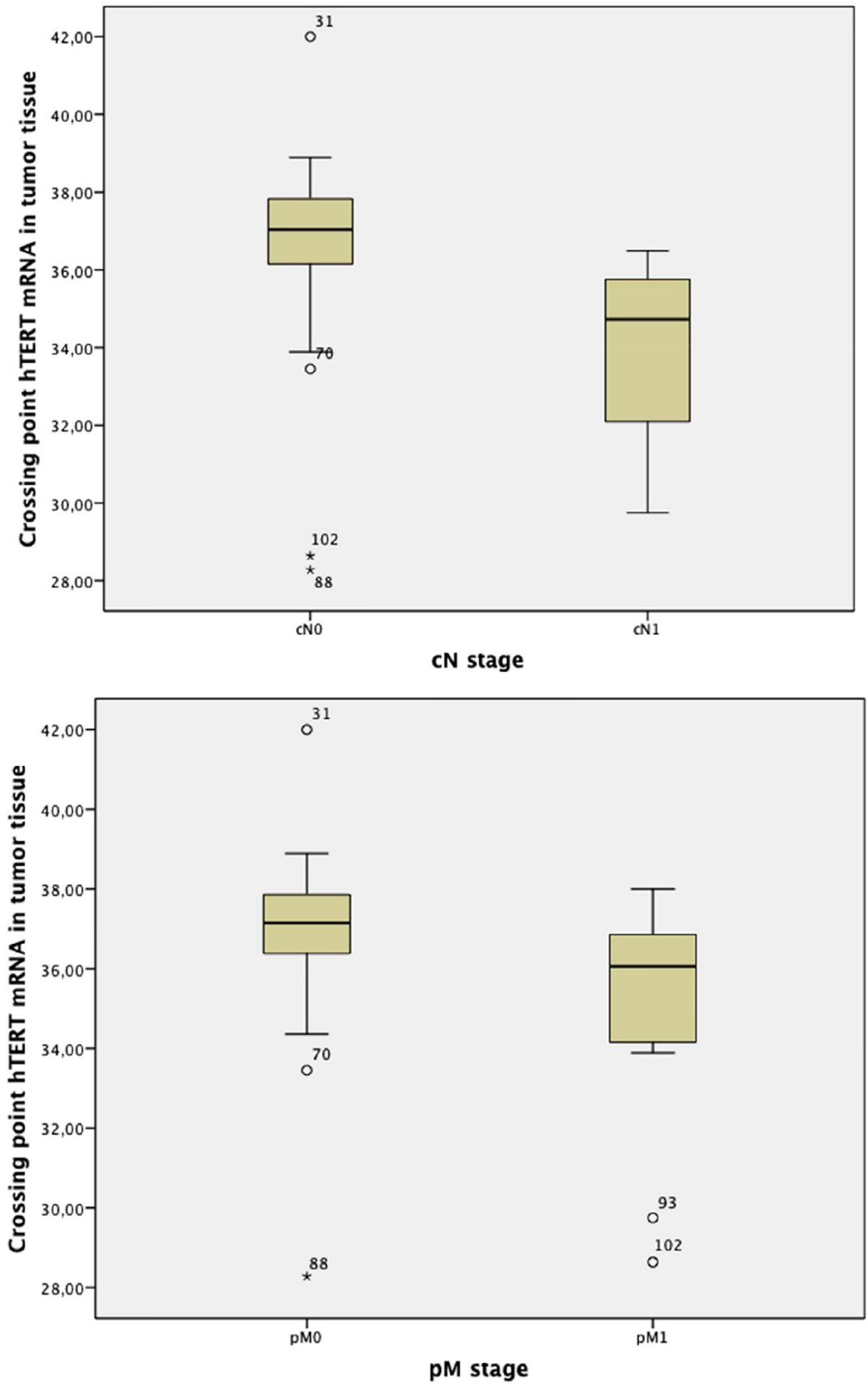

Fig. 2. Crossing point hTERT mRNA in tumor tissue in cN ($P = 0.018$) and pM stages ($P = 0.046$).

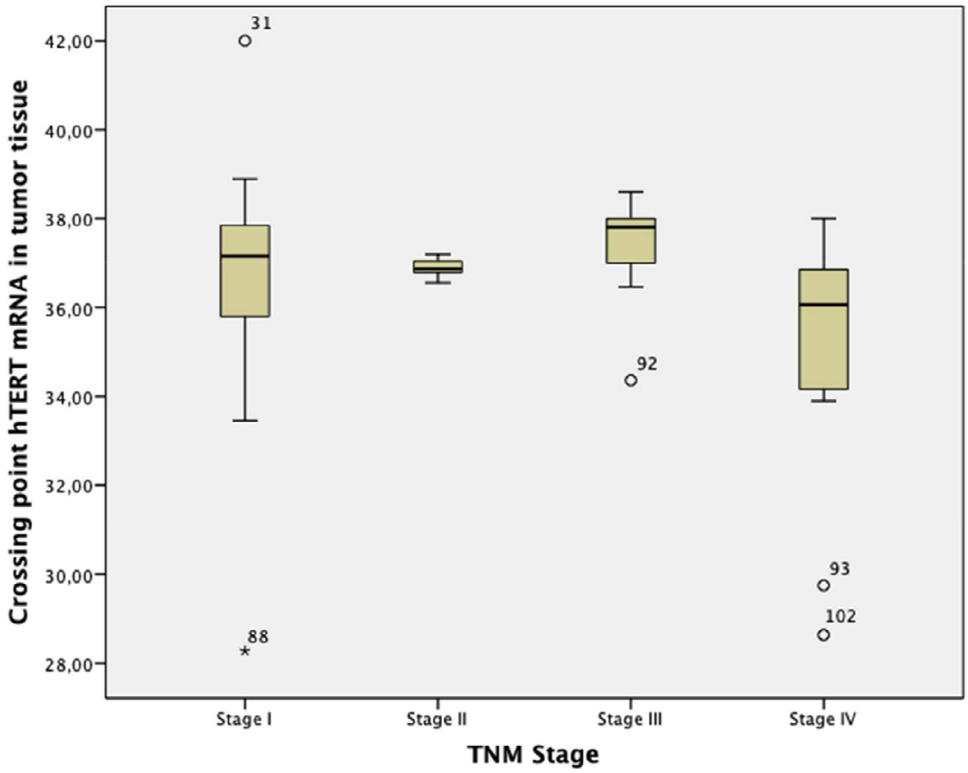

Fig. 3. Crossing point hTERT mRNA in tumor tissue in TNM stage ($P = 0.041$).

With respect to TNM stage, no significant differences were found in the expression of hTERT mRNA in tumor tissue between the different clinical stages, although it was observed that the CP of hTERT mRNA was significantly lower in stage IV compared to earlier stages. This information, together with that obtained in our series on lymph node involvement and distant metastasis, leads us to consider the prognostic potential of this marker. Our results confirm those of previous studies [9], in which greater expression of hTERT mRNA has been described in more advanced TNM stages, although without showing statistical significance. This suggests, nevertheless, that expression of hTERT mRNA is related to the aggressiveness of the RCC. Finally, studies on gastric cancer [15] have also found a relationship between expression of hTERT mRNA and advanced clinical stages and lymphatic invasion.

A recent publication, [16] analyzes the expression of hTERT protein by means of immunohistochemistry, showing

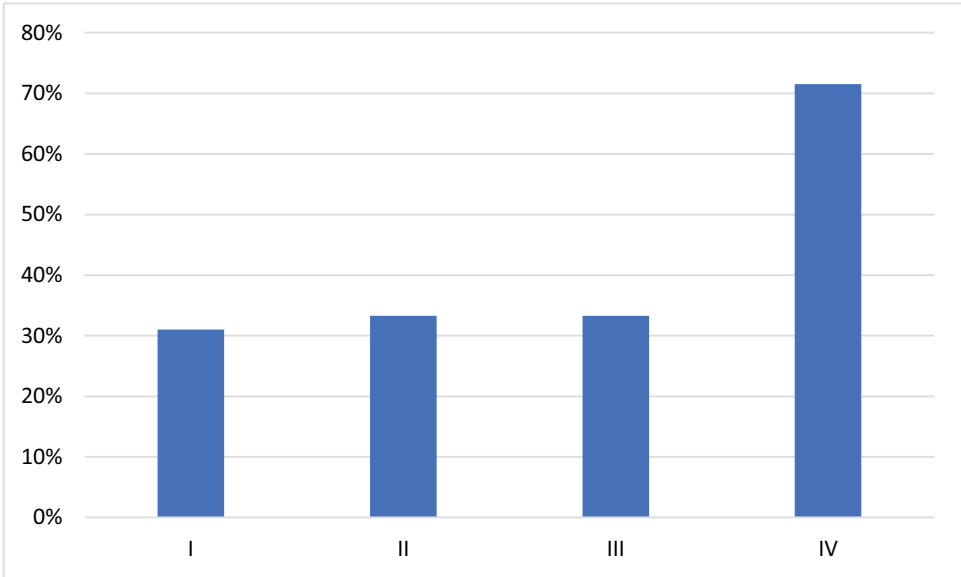

Fig. 4. Percentages of 3 hTERT mRNA replicates according to TNM stage.

Table 4
Number of hTERT mRNA replicates according to histopathological variables

Histopathological variables		hTERT mRNA replicates				*P*
		0 *n* (%)	1 *n* (%)	2 *n* (%)	3 *n* (%)	
Histology	Clear cell	17 (22.1)	20 (26.0)	10 (13.0)	30 (39.0)	0.002
	Papillar	4 (30.8)	3 (23.1)	0 (0.0)	6 (46.1)	
	Chromophobe	7 (63.6)	3 (27.3%)	0 (0.0)	1 (9.1)	
	Sarcomatoid	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	
	Oncocytomas	7 (87.5)	1 (12.5)	0 (0.0)	0 (0.0)	
Fuhrman degree	1	3 (42.8)	1 (14.3)	1 (14.3%)	2 (28.57)	NS
	2	14 (29.8)	9 (19.14)	5 (10.6%)	19 (40.4)	
	3	5 (18.5)	12 (44.4)	3 (11.1%)	7 (25.9)	
	4	2 (16.7)	2 (16.7)	1 (8.3%)	7 (58.3)	
pT	1	18 (31.6)	14 (24.6)	7 (13.0%)	18 (31.6)	NS
	2	4 (25.0)	6 (37.5)	0 (0.0)	6 (37.5)	
	3	3 (13.6)	6 (27.3)	2 (9.1)	11 (50.0)	
	4	1 (20.0)	0 (0.0)	1 (20.0)	3 (60.0)	
N	0	24 (25.5)	26 (27.6)	10 (10.6)	34 (36.3)	NS
	1	1 (20.0)	0 (0.0)	0 (0.0)	4 (80.0)	
pM	0	27 (30.0)	25 (27.8)	9 (10.0)	29 (32.2)	0.005
	1	1 (7.7)	1 (7.7)	1 (7.7)	10 (84.6)	
TNM stage	I	19 (32.7)	14 (24.1)	7 (12.1)	18 (31.0)	0.017
	II	4 (26.7)	6 (39.1)	0 (0.0)	5(33.3)	
	III	4 (26.7)	5 (33.3)	1 (6.7)	5 (33.3)	
	IV	1 (7.1)	1 (7.1)	2 (14.3)	10 (71.5)	

results that are coherent with ours. The authors find a greater expression of hTERT protein with the ccRCC histological subtype and that in addition, it is also associated with those cases in which ccRCC had a more aggressive behavior and in patients with more advanced disease.

Regarding expression of GAPDH mRNA in paired tumor and normal tissue, it was present in all samples, although the CP in RCC cases was significantly lower than in normal tissue samples. This finding is remarkable, since GAPDH is a housekeeping gene whose expression is stable and should not be altered according to clinical status.

Some authors [17] have found varying levels of GAPDH expression in ccRCC samples compared to their more stable expression in paired healthy tissue, from which they concluded that each tumor is unique and may present genomic instability responsible for modifications in the expression of housekeeping genes. Although we share this view in the present study, the greater cellularity associated with tumor tissue may also alter GAPDH expression in tumor samples with respect to healthy tissue.

In conclusion, the expression of hTERT mRNA is a verifiable phenomenon pertaining to RCC. Its greater expression in certain types of renal tumors, such as sarcomatoid and clear cell, together with higher levels of expression in more advanced cases of RCC, signify that determination of hTERT mRNA levels in tissue can serve as a marker for histological diagnosis with prognostic value. Studies with a larger patient series and ample follow-up time are needed to confirm our findings.

Conflict of interest

The authors do not declare conflict of interest.

Acknowledgments

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