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Original Research

Clinical value of perioperative levels of DNA and mRNA in plasma of patients with renal cell carcinoma

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ABSTRACT

Introduction: The current challenge on renal cell carcinoma (RCC) is to finding a non-invasive biomarker for improving their diagnostic and therapeutic management. In the present study, we analyzed the clinical value of plasma levels of cell-free DNA (cfDNA) and RNA (cfRNA) of two genes: glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) and human telomerase reverse transcriptase (h*TERT*).

Materials and methods: We recruited 82 patients with RCC, and 20 healthy subjects. Using RT-PCR techniques, plasma levels of cfDNA and cfRNA from h*TERT* and *GAPDH* genes were quantified pre- and post-operatively, and one year after surgery. Relationships between such plasma levels and clinicopathological features and evolution of disease were analyzed.

Results: Levels of *GAPDH* cfDNA and cfRNA were significantly higher in patients than in healthy subjects. h*TERT* cfDNA was detected in plasma from 35% of RCC patients and in none healthy subject. At diagnosis, plasma levels of *GAPDH* cfDNA were higher in advanced pT and TNM stages, and h*TERT* cfDNA in patients with 3–4 Fuhrman grade and affected lymph nodes. Levels of cfNAs were not related to the presence of metastasis. Following nephrectomy, *GAPDH* cfDNA levels dropped, and patients with higher levels before and after nephrectomy, showed lower overall survival (OS). However, Cox's multivariate model did not prove any association of the cfNA levels with progression.

Conclusion: Plasma levels of cfDNA from *GADPH* and h*TERT* genes were correlated to tumor diagnosis and progression and, thus, such analyses might help to diagnosis and prognosis of RCC patients.

Introduction

Renal cell carcinoma (RCC) is the most common solid lesion of the kidney and accounts for approximately 90% of all malignant renal tumors. There are three different types of RCC, with specific histopathological and genetic characteristics [1]; the most frequent (80–90%) is the clear cell carcinoma (ccRCC), followed by papillary and chromophobe (15% and 5%, respectively) carcinomas. RCC is a difficult entity for clinical management, mainly due to a heterogeneous course among patients, which is related to histopathological and molecular heterogeneity.

The diagnostic and prognostic guidelines for this type of cancer are only based on the clinicopathological history (TNM stage, Fuhrman grade, histological type and molecular features) and radiological examinations, due to the lack of blood and urine biomarkers. The search of specific markers appears to be essential for improving the management of RCC patients along disease phases: from diagnosis to treatment and follow-up [2].

One of the current challenges in the research of metastatic renal cancer is to find a blood biomarker for predicting prognosis and optimizing the therapeutic approaches [3]. Circulating tumor cells (CTCs) have been proposed as biomarkers of cancer disease, with potential application for early diagnosis, staging, choice of therapeutic approach, follow-up, and prognosis [4]. It has been reported that CTCs were detected up to 94% of RCC patients [4] and it was linked to a poor prognosis [5]. However, there are not yet enough evidences to introduce such technique into clinical routine.

On the other hand, quantitation of cell-free nucleic acids (cfNA) circulating in plasma appears to be easier and more reproducible than de-

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tection of CTCs [6]. However, many factors can influence the results of cfNA analysis, such as the technique employed, the genetic profile analyzed, the tumor stage, or the grade of tumor heterogeneity. Cell-free DNA (cfDNA) is usually found as single-stranded fragments in plasma, and rates of detection of tumor cfDNA reported for cancer patients have been very variable among studies (between 0.01% and 93%) [7]. With respect to circulating cell-free RNA (cfRNA), results of many previous studies have been unexpected, because such RNA has been detected with relative easiness, in spite of the increased RNAse levels in plasma of cancer patients [7]. It is probably than the unexpected stability of those molecules might be related to vesicular structures protecting it [7].

One of the cfRNA that has been most frequently analyzed in plasma has been the transcript of the human telomerase reverse transcriptase gene (h*TERT*) [8–10]. Telomerase is a ribonucleoprotein complex that contains an internal RNA template and a catalytic protein with telomere-specific reverse transcriptase activity. It is involved in the repair and lengthening of telomeres at the end of eukaryotic chromosomes, preventing cell senescence and death, and it is overexpressed in a variety of tumors [11–13]. In fact, a previous own study on RCC tumor tissue showed expression of h*TERT* mRNA in those tissues, and the expression levels were correlated with histopathologic diagnosis and TNM stages [14]. Such overexpression was in agreement with results of previous studies in other kind of cancers [10,12,14] and might be reflected in the release of cell-free mRNA into plasma. In fact, levels of h*TERT* mRNA sequences in plasma have been reported as a potential tool for the diagnosis and monitoring of such cancers [8–13,15].

Transcripts of housekeeping genes, such as the gene for glyceraldehyde3-phosphate-dehydrogenase (*GAPDH*), have also been detected in plasma [10,16]. Quantitation of such transcripts has served, in many studies, as an internal control or to normalize the expression of other RNAs. However, some previous studies have shown the potential utility of such quantitation as an independent biomarker for cancer disease, *e.g.* for larynx cancer [10].

The goal of the present study was to evaluate the diagnostic and prognostic utility of perioperative detection and quantitation of cfDNA and cfRNA sequences of *GAPDH* and h*TERT* genes in plasma from patients with RCC.

Materials and methods

We performed a prospective, longitudinal study of a cohort of 82 patients diagnosed with RCC, who had undergone surgery in the Urology Department of the University General Hospital of Albacete (Spain). The study was approved by the hospital's Ethics Committee (code: 04/2015). The recruitment of patients began in March 2015 and finished in October 2018.

The following variables were collected from patients: age, sex, tumor size, type of surgery (total or partial nephrectomy, open or laparoscopic surgery), TNM stage, Fuhrman grade, tumor histology and perioperative complications.

Additionally, 20 healthy volunteers were recruited. They were accompanying of patients who were being treated in another department of our hospital. The inclusion criterion was absence of oncological disease, which was verified through the medical records of the volunteer in our hospital.

Patients and healthy volunteers gave their written consent to be included in the present study.

Blood sampling

Three blood samples were collected from each patient, at three different times: (i) preoperatively, in the operating room immediately before surgical resection; (ii) postoperatively, at the day of the patient discharge; and (iii) one year after surgery.

Only one blood sample was collected from each healthy volunteer.

Each blood sample had 8–10 mL, and was collected in a tube with EDTA. All samples were centrifuged immediately after collection at 2000 × 3 g for 10 min. Plasma was collected and centrifuged a second time at 3000 × 3 g for 10 min. Centrifugations were performed at 4 °C, and plasma samples were then frozen at −80 °C until analysis.

Extraction of cell-free DNA from plasma

DNA was extracted from 1-ml samples of plasma, with a commercial kit (Danagen circulating DNA minikit, Danagen-Biotech®, Barcelona, Spain), which enables the collection of high-quality circulating DNA. Once extracted, DNA samples were frozen at −20 °C.

Extraction of cell-free RNA from plasma

RNA was extracted from 1-mL samples of plasma. Firstly, cfNAs were extracted with a commercial kit (QIAmpUltraSens Virus; Qiagen, Venlo, The Netherlands). Then, samples were subjected to DNase digestion and subsequent retrotranscription with a commercial kit (QuantiTect Reverse Transcription Kit; Qiagen, Venlo, The Netherlands). To confirm the absence of DNA in our RNA samples before reverse transcription, we performed a conventional PCR to amplify a 226-bp sequence of the gene for *GAPDH*. After retrotranscription, cDNA samples were frozen at −20 °C.

Quantitation of DNA and cDNA

Samples of DNA and cDNA were analyzed by two real-time PCRs with fluorescent hybridization probes for amplification, respectively, of *GAPDH*-specific and h*TERT*-specific sequences.

In the case of GAPDH, we amplified a 226-bp sequence with forward and reverse primers 5′-gaaggtgaaggtcggagtc-3′ and 5′-gaagatggtgatgggatttc-3′. We used a TaqMan® probe (see below for source) 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 tetramethyl rhodamine (TAMRA) to yield the following oligonucleotide: 5′-FAM-caagcttcccgttctcagcc-TAMRA. In the case of h*TERT*, we amplified a 95-bp sequence with the forward and reverse primers 5′-tgacacctcacctcacccac-3′ and 5′-cactgtcttccgcaagttcac-3′. The h*TERT*-specific TaqMan® probe had the following sequence: 5′-FAM-accctggtccgaggtgtgtccctgag-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, Mannheim, Germany). The reaction mixture, with a total volume of 20 µl, contained 2 µl of the solution of cDNA plus 0.5 µM each primer, 0.2 µM probe and 3 mM $MgCl_2$.

In all amplification experiments, DNA from renal tumors and water were included as positive and negative controls, respectively. DNA from plasma of healthy subjects was also included as a positive control for amplification of GAPDH sequences.

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 min. 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 h*TERT* cDNA. The first step in the amplification was incubation for 15 s at 95 °C. The annealing/extension incubation was allowed to proceed for 30 s at 59 °C in the case of GAPDH cDNA and for 30 s at 65 °C for h*TERT* 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. We generated four standard curves for the quantitation of, respectively, 226-bp GAPDH-specific

sequences and 95-bp h*TERT*-specific sequences. Each curve was created by plotting the logarithm of the amount of DNA against the threshold cycle number. The amounts of DNA or cDNA in samples to be analyzed were determined from the appropriate standard curves.

The values obtained by the above-described method were recalculated to generate concentrations (fg/ml of plasma).

Statistical analysis

We performed a descriptive analysis of all the variables, which included bivariate analysis using contingency tables for qualitative variables, and comparisons of means between independent groups for quantitative ones. Nonparametric tests were used when the assumptions of normality were not fulfilled. Pearson´s test was applied to measure the relationship between two quantitative and continuous variables.

To evaluate the performance of the diagnostic tests, we calculated the ROC curves for each marker used by calculating its sensitivity and specificity values.

Using survival analysis (Kaplan-Meier life table method), the probability of survival at the end of each follow-up period, was calculated. Overall survival (OS) and cancer-specific survival (CSS) were recorded from the nephrectomy until *exitus* or end of study. Progression-free period (PFS) was calculated from the date of surgery until diagnosis of clinical or radiological progression. Both mean and median survival duration, as well as confidence intervals, were calculated. Then, survival curves and comparisons were made using Mantel-Haenszel (logrank) test.

Lastly, we performed a univariate and multivariate Cox proportional hazards analysis. The SPSS v. 20.0. Statistical package was employed for the entire statistical analysis (Armonk, NY, IBM Corp, USA)

Results

Descriptive analysis

We analyzed 82 RCC patients and 20 healthy volunteers. In the patient cohort, the 68.3% were men, and the mean age was 59.7 years (SD:13.05). The healthy volunteers were 85% men and the mean age was 59.5 years (SD:12.4). Differences were not statistically significant.

Results of clinicopathologmk jnhhhhhhhhunb ical characteristics of patients were detailed in Table 1. In summary, most of patients had clear-cell renal tumors (ccRCC), and the most frequent stages were pT2 or lower, and Fuhrman grade 2 or lower. Distant metastases at diagnosis were infrequent.

Diagnostic value of plasma levels of cfDNA and cfRNA

GAPDH cfDNA sequences were found in all plasmas analyzed. We found statistically significant differences between RCC patients and healthy volunteers in plasma levels of *GAPDH* cfDNA. Specifically, the mean of such levels was 29.3 fg/ml (SD: 133.4) in RCC patients, and 1.3 fg/ml (SD: 5.1) in heathy volunteers ($p< 0.0001$). Similar differences were found when we compared *GAPDH* cfRNA levels: 146.6 fg/ml (SD: 422.0) in patients *versus* 20.6 fg/ml (SD: 27.4) in healthy volunteers ($p< 0.0001$).

For a prevalence of 76.2%, the diagnostic sensitivity of *GAPDH* cfDNA levels above 1.3 fg/ml, was 39.1%, and the specificity was 90%. The area under the curve (AUC) was 0.861 (0.755–0.068; $p< 0.0001$). For a prevalence of 75.6%, the RCC diagnostic sensitivity for *GAPDH* cfRNA levels higher than 20.6 fg/ml, was 21.0%, with 70% specificity. The area under the curve was 0.231 (CI 95%: 0.131–0.332; $p< 0.0001$). h*TERT* cfDNA was detected in plasma from only 28 RCC patients (34.6%), with a mean value of 0.93 fg/ml (SD: 3.0), whereas none of the control analyses showed traces of this marker.

With respect to h*TERT* cfRNA, we analyzed plasma samples from 21 out of the 82 cancer patients and detection of such sequences was positive in only one sample. That result led us to discontinue the analysis. In addition, h*TERT* cfRNA was not detected in any plasma sample from healthy volunteers.

Table 1
Results of main clinicopathological characteristics of cancer disease in RCC patients recruited in this study ($n = 82$).

MULTIPLICITY (%)	
Single	76 (92.7)
Multiple	6 (7.3)
MEAN DIAMETER (cm)	6.08 (SD:3.38)
pT STAGE (%)	
pT1-pT2	60 (73.2)
pT3-pT4	22 (26.8)
pM STAGE (%)	
pM0	70 (85.4)
pM1	12 (14.6)
cN STAGE (%)	
cN0	78 (95.1)
cN1	4 (4.9)
TNM STAGE (%)	
I-II	58 (70.7)
III-IV	24 (29.3)
HISTOLOGY (%)	
Clear cell	67 (81.7)
Papillary	8 (9.8)
Chromophobe	6 (7.3)
Sarcomatoid	1 (1.2)
FUHRMAN GRADES (%)	
1–2	45 (70.7)
3–4	27 (29.3)
PERIOPERATIVE COMPLICATIONS (%)	
No	75 (91.5)
Yes	7 (8.5)
***EXITUS* during follow-up** (%)	
No	73 (89.0)
Yes	9 (11.0)
CONDITION at the end of the follow-up (%)	
In remission	59 (72.0)
Progression or exitus	23 (28.0)

Prognostic value of preoperative plasma levels of cfDNA and cfRNA

Regarding RCC patients, Table 2 shows the mean levels of cfNA detected in plasma samples, and the main findings of the statistical analysis.

Briefly, at diagnosis, *GAPDH* cfDNA and cfRNA plasma levels were correlated to tumor diameter and, in addition, the mean level was higher in patients with pT3-pT4 and with TNM III-IV stages. h*TERT* cfDNA plasma levels were higher in patients with N1 stage and with Fuhrman grades 3–4.

Prognostic value of postoperative plasma levels of cfDNA and cfRNA

After nephrectomy, the mean level of *GAPDH* cfDNA in plasma was 16.9 fg/ml (SD: 28.1), which was significantly lower than preoperative result ($p< 0.0001$). Postoperative plasma levels of *GAPDH* cfDNA were not correlated with any clinical variable

Postoperative plasma levels of *GAPDH* cfRNA and h*TERT* cfDNA were similar to preoperative levels.

Prognostic value of plasma levels of cfDNA and cfRNA one year after surgery

One year after surgery, plasma levels of all cfNA tested were similar to those found at preoperative.

In plasma samples extracted one year after surgery from patients with ccRCC, we detected a mean level of *GAPDH* cfDNA that was higher than those found with other RCC histological types: 386.0 fg/ml (SD: 1265.7) *versus* 81.4 fg/ml (SD: 35.6; $p = 0.029$).

Table 2
Results of preoperative quantitation of cell-free DNA and RNA in plasma from RCC patients, and statistical relationship between principal clinicopathological variables.

	GAPDH DNA		*hTERT* DNA		*GAPDH* RNA	
	r	p	r	p	R	p
TUMOR DIAMETER (cm)	0.273	0.013	−0.016	N.S.	0.258	0.043
	Mean (SD) fg/ml	**p**	**Mean (SD)** fg/ml	**p**	**Mean (SD)** fg/ml	**p**
MULTIPLICITY						
Single	14.9 (55.1)	N.S.	0.99 (3.1)	N.S.	135.6 (411.0)	N.S.
Multiple	167.6 (405.3)		0.42 (0.8)		271.5 (580.9)	
pT STAGE		**0.033**				
pT1-pT2	12.1 (67.8)		0.96 (3.4)	N.S.	131.3 (391.4)	N.S.
pT3-pT4	76.9 (239.1)		0.85 (1.2)		190.5 (513.8)	
pM STAGE						
pM0	30.9 (145.7)	N.S.	0.96(3.2)	N.S.	84.1 (285.7)	N.S.
pM1	21.2 (40.5)		0.88 (1.3)		436.4 (754.4)	
cN STAGE						
cN0	29.5 (137.4)	N.S.	0.89 (3.0)	**0.023**	128.1 (386.6)	N.S.
cN1	25.5 (48.8)		1.55 (1.7)		415.3 (829.8)	
TNM STAGE						
I-II	12.5 (59.0)	**0.033**	1.01 (3.5)	N.S.	96.4 (306.2)	N.S.
III-IV	68.9 (226.7)		0.76 (1.2)		269.4 (617.5)	
FUHRMAN						
Grades 1–2	4.4 (10.7)	N.S.	3.2 (0.5)	**0.032**	111.6 (327.4)	N.S.
Grades 3–4	59.6 (211.4)		2.9 (0.6)		230.9 (577.5)	
HISTOLOGY						
Clear cell	28.0 (138.7)	N.S.	1.1 (3.2)	N.S.	23.9 (78.3)	N.S.
Non-clear cell	34.7 (112.7)		0.5 (1.2)		173.1(461.1)	
EXITUS during follow-up						
No	29.6 (141.7)	N.S.	0.9 (3.1)	**0.018**	137.3 (399.3)	N.S.
Yes	26.5 (46.9)		1.1 (1.4)		207.7 (586.8)	
CONDITION AT THE END OF FOLLOW-UP						
In remission	14.0 (59.4)	N.S.	1.0 (3.4)	N.S.	66.5 (242.5)	N.S.
Progression or *exitus*	65.3 (227.3)		0.7 (1.1)		342.2 (659.6)	

N.S.: not significative.

In addition, in patients with pT3-pT4 tumors, plasma levels of h*TERT* cfDNA were higher than those found in patients with pT1-pT2 tumors: 4.3 fg/ml (SD: 9.4) *versus* 0.7 fg/ml (SD: 2.5; $p = 0.006$).

Plasma levels of *GAPDH* cfRNA detected one year after surgery were not correlated with any clinical variable.

Follow-up

The mean follow-up time was 33.72 months (CI 95%: 30.6–36.96), with a minimum of 3.36 months, and a maximum of 57.24. The median follow-up time was 32.64 months (SD: 14.52). At the end of the study, in 23 patients (28.0%) the disease had progressed and 9 of them (9/82, 11%) had died (Table 1). Most of those deaths (8/9) were due to cancer disease.

The probability of cumulative survival was 97.5% at the end of the first year, and 87.5% by the end of the third year, with no deaths occurring after this time.

The PFS was 37.0 months (CI 95%: 28.4–35.6), with a minimum of 0.3 months and a maximum of 58.1 months. The median PFS was 31.2 months (SD: 16.5).

The cumulative probability of PFS was 91.0% at the end of the first year, and 86.0% at the end of the third one, with no cases of further progression after this time.

Relationship between quantitation of cfNA and survival

Table 3 shows results of Cox regression analyses for OS and disease progression with respect to preoperative variables.

Briefly, the univariate Cox regression analysis showed that the variables related to the lowest OS were the presence of metastasis at diagnosis and stages TNM III-IV. However, in the multivariate model, in addition to such detection, the preoperative quantitation of *GAPDH* cfDNA was statistically related to OS. Specifically, for each fg/ml of GAPDH cfDNA, the risk of death was multiplied by 1.04 (Table 3).

In addition, the presence of metastasis, stages TNM III-IV and preoperative levels of *GAPDH* and h*TERT* cfDNA were associated with a greater risk of disease progression. Thus, for every fg/ml of *GAPDH* cfDNA at diagnosis, the risk of progression was multiplied by 1.01, and for every fg/ml of h*TERT* cfDNA, by 1.23. However, in the multivariate model, the only variable associated with progression risk was the presence of metastasis at diagnosis (Table 3).

Table 4 shows results of Cox regression analyses for OS and disease progression with respect to postoperative analyses. The univariate analysis showed that a OS was decreased in patients with metastasis at diagnosis, with advanced tumor stages (TNM III-IV), and with high post-operative levels of *GAPDH* cfDNA in plasma. All of these variables (except for TNM stage) remained in the multivariate model, with the incorporation of non-clear-cell tumors. Thus, the risk of dying was increased by 1.029 for every fg/ml of *GAPDH* cfDNA detected in plasma after surgery, by 0.16 for non-clear-cell tumors, and by 14.8 for patients with metastasis at diagnosis (Table 4).

In the univariate model, the lowest PFS were related to presence of metastasis, advanced tumor stages (TNM III-IV), and post-operative levels of *GAPDH* and h*TERT* cfDNA in plasma. We found that for every fg/ml of plasma *GAPDH* cfDNA and of plasma h*TERT* cfDNA at post-operative time, the risk of progression was increased by 1.01 and by 1.23, respectively. However, in the multivariate model, postoperative

Table 3
Results of univariate and multivariate statistical analyses for identifying the variables that could predicted preoperatively overall survival and disease progression in RCC patients. Pre-op: preoperative.

Overall survival Univariate Cox model	p	Exp(B)	CI95%
Age	0.114	1.05	0.98–1.11
Histology			
1No ccRCC		1 (ref)	
Yes ccRCC	0.16	0.36	0.86–1.52
pM stage			
pM0		1 (ref)	
pM1	< **0.0001**	22.5	4.50–112.5
TNM stage			
TNM I-II		1(ref)	
TNM III-IV	**0.03**	3.44	2.95–201.6
Pre-op *GAPDH* cfDNA	0,08	1.01	1.003–1.029
Pre-op *GAPDH* cfRNA	0.46	1.00	0.99–1.00
Pre-op *hTERT* cfDNA	0.6	1.02	0.83–1.26
Multivariate Cox model			
pM stage			
pM0		1(ref)	
pM1	< **0.0001**	20.4	3.8–109.9
Pre-op *GAPDH* cfDNA	**0.01**	1.04	1.01–1.07
PROGRESSION Univariate Cox model	p	Exp(B)	CI95%
Age	0.67	1.01	0. 97–1.041
Histology			
No ccRCC		1 (ref)	
Yes ccRCC	0.67	1.30	0.38–4.5
pM stage			
pM0		1 (ref)	
pM1	< **0.0001**	36.6	1.52–107.5
TNM stage			
TNM I-II		1(ref)	
TNM III-IV	< **0.0001**	8.9	3.32–23.8
Pre-op *GAPDH* cfDNA	**0.01**	1.01	1.001–1.021
Pre-op *GAPDH* cfRNA	0.67	1.00	0.99–1.00
Pre-op *hTERT* cfDNA	< **0.0001**	1.23	1.09–1.32
Multivariate Cox model			
pM stage			
pM0		1(ref)	
pM1	< **0.0001**	97.7	18.5–515.5

Table 4
Results of univariate and multivariate statistical analyses for identifying the variables that could predicted postoperatively overall survival and disease progression in RCC patients. Post-op: postoperative.

OVERALL SURVIVAL Univariate Cox model	p	Exp(B)	CI95%
Age	0.114	1.05	0.98–1.11
Histology			
No ccRCC		1 (ref)	
Yes ccRCC	0.11	0.36	0.86–1.52
pM stage			
pM0		1 (ref)	
pM1	< **0.0001**	22.5	4.49–113.5
TNM stage			
TNM I-II		1(ref)	
TNM III-IV	**0.003**	24.4	2.95–201.6
Post-op *GAPDH* cfDNA	**0,01**	1.01	1.003–1.029
Post-op *GAPDH* cfRNA	0.15	1.00	0.99–1.002
Post-op *hTERT* cfDNA	0.24	1.09	0.64–1.27
Multivariate Cox model			
Histology			
No ccRCC		1 (ref)	
Yes ccRCC	**0.046**	0.169	0.029–0.967
pM stage			
pM0		1(ref)	
pM1	**0.002**	14.8	2.7–81.7
Post-op *GAPDH* cfDNA	**0.01**	1.029	1.005–1.05
PROGRESSION Univariate Cox model	p	Exp(B)	CI95%
Age	0.67	1.01	0. 97–1.041
Histology			
No ccRCC		1 (ref)	
Yes ccRCC	0.67	1.30	0.38–4.5
pM stage			
pM0		1 (ref)	
pM1	< **0.0001**	36.6	1.52–107.5
TNM stage			
TNM I-II		1(ref)	
TNM III-IV	< **0.0001**	8.9	3.32–23.8
Post-op *GAPDH* cfDNA	**0.01**	1.01	1.001–1.021
Post-op *GAPDH* cfRNA	0.67	1.00	0.99–1.00
Post-op *hTERT* cfDNA	< **0.0001**	1.23	1.09–1.32
Multivariate Cox model			
pM stage			
pM0		1(ref)	
pM1	< **0.0001**	97.7	18.5–515.5

levels of *GAPDH* and h*TERT* cfDNA were not independent risk factors for progression, but only the presence of metastasis at diagnosis was (Table 4).

Levels of cfDNA and cfRNA detected one year after surgery were not statistically related to OS nor PFS.

Discussion

The potential clinical value of plasma cfNA in diagnosis of RCC has been evaluated by several approaches. To date, no genetic alterations common to a majority of RCC have been found and, for this reason, detection of tumor cfNA in plasma from those patients requires sophisticated and expensive techniques. To circumvent such disadvantage, it has been evaluated some alternative strategies based on total (tumor and non-tumor) cfNA. For example, it has been shown that the quantity of medium and small-sized cfDNA fragments did not differ between RCC patients and healthy volunteers and, by contrast, larger fragments were more frequent in patients and, particularly, in metastatic patients [17–19]. In addition, plasma levels of housekeeping genes, such as beta-actin, has been also proposed as complementary tools for diagnosis of RCC [20].

The literature shows variable percentages of sensitivity and specificity of analyses of cfNA in plasma for diagnosis of RCC: from 25% to 78% [3,18]. Those apparent discrepancies might be related to a variety of reasons, such as the clinical heterogeneity of RCC patients, the diversity of methods used, or even the design of primers sequences [3].

In the current study we evaluated the potential utility of detection in plasma of both cfDNA and cfRNA sequences from two genes: *GADPH* and h*TERT*. To our knowledge, it is the first time that those molecules have been detected in plasma from RCC patients. We found that levels of *GAPDH* cfDNA and cfRNA in plasma were significantly increased in RCC patients respect to a group of healthy volunteers and, moreover, that detection of such high levels might serve as diagnostic tools for RCC, with a sensitivity up to 39% and a specificity up to 90%. Detection of h*TERT* cfDNA was not possible in any healthy volunteer, but in 35% of RCC patients, which might confer some diagnostic value, at least as a complementary method.

By contrast, h*TERT* cfRNA was not a marker useful for diagnosis nor prognosis of RCC in the present study, because the rate of detection in plasma from cancer patients was extremely low (5%). This result was unexpected, because, with an identical technique, some previous studies have reported the utility of this potential biomarker in other kinds of cancer, such as colorectal or laryngeal cancer [9,10,13]. In addition, an own previous study in RCC patients showed expression of hTERT mRNA in 68% of tumor tissues, which was significantly correlated with histopathology and tumor TNM stage [14]. Further studies are needed to

clarify the reason why such h*TERT* expression is undetectable in plasma using a method which suitability has been demonstrated in previous studies [10].

In the current study, we did not find any relationship between plasma levels of cfNA and histological classification of tumors, which supports other recent data [21,22]. In addition, although it has been previously suggested that levels of plasma cfNA levels are related to metastatic disease [3,22], the current study showed a striking absence of such relationship. The heterogeneity and particular biology of RCC tumors might be the reason for those apparent contradictory results. In fact, that argument has been proposed as the reason of the low rate of detection of CTCs in patients with metastatic RCC [23].

By contrast, plasma levels of *GAPDH* cfDNA were increased in patients with tumors in pT and TNM advanced stages, and *GAPDH* cfRNA levels were increased in patients with tumors with advanced Fuhrman grades and lymphatic dissemination. These results supported those of previous studies that showed cfDNA levels according to renal tumor stage [20,24]. Thus, it appears that preoperative quantitation of cfNA sequences from housekeeping genes might have any utility as prognostic markers. Moreover, we found a positive correlation between tumor size and *GAPDH* cfDNA and cfRNA levels. Previous studies on RCC have suggested a relationship between necrosis and higher cfDNA levels [19], which might be a reason for our results, since tumors of greater volume display larger areas of necrosis.

Afterwards nephrectomy and one year later, plasma levels of cfRNA did not give any information about prognosis. However, cfDNA plasma levels variations showed a potential utility for prognosis and follow-up. Specifically, levels of *GAPDH* cfDNA suffered a significant decrease, which was in agreement with previous studies on patients under curative nephrectomy [18]. In fact, it has been suggested that the reduction of cfDNA after nephrectomy might be an independent prognostic factor that is also associated with lower risk of recurrence [25]. However, one year after surgery, ccRCC patients in the current study showed an increase in the *GAPDH* cfDNA plasma levels, which also occurred in patients with pT3-pT4 tumors.

With respect to survival, although patients who died showed increased plasma levels of h*TERT* cfDNA at diagnosis, only pre and postoperative levels of *GAPDH* cfDNA were independently associated with OS, but not with PFS. Some clinical features were confirmed as independent risk factors for poorer OS and PFS; specifically, detection of metastases and TNM stages III-IV on diagnosis. Previous studies have shown a that post-nephrectomy levels of cfDNA in plasma had a direct relationship with later recurrence [24]. In the present study, quantitative analysis of plasma cfNA in the early postoperative gave results with some prognosis value, however, in the late postoperative period (one year) such analyses were not useful to predict survival.

Summarizing, the results of the present study are in addition to those of previous studies focused on the utility of cfNA in the diagnosis and management of RCC patients [3,17–19,26]. Quantitation of cfNA in plasma (particularly, cfDNA) has promising perspectives as RCC biomarker, although it appears necessary to combine several plasma markers to obtain useful applicable to clinical routine.

To conclude, the results of the current study show that levels of cfDNA from *GADPH* and h*TERT* genes were correlated to tumor progression and, thus, such analyses might help to diagnosis and prognosis in RCC patients.

Declaration of Competing Interest

The authors do not declare conflict of interest.

CRediT authorship contribution statement

Antonio S. Salinas-Sánchez: Conceptualization, Funding acquisition, Writing - original draft. **Dolores C. García-Olmo:** Conceptualization, Funding acquisition, Writing - original draft, Supervision. **Carlos Martínez-Sanchiz:** Methodology, Resources, Visualization. **María G. Picazo-Martínez:** Formal analysis. **José M. Giménez-Bachs:** Data curation, Validation. **Ana B. Flores-Bautista:** Investigation. **Ángela Díaz-Piqueras:** Formal analysis.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100999.

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