Cytoplasmic PAR-3 protein expression is associated with adverse prognostic factors in clear cell renal cell carcinoma and independently impacts survival.

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TITLE PAGE

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Keywords

PAR-3 protein; renal cell carcinoma; biomarker; prognosis; survival

Runninghead

PAR-3 in clear cell renal cell carcinoma prognosis

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ABSTRACT

Clear cell renal cell carcinomas (ccRCC) represent 70% of renal cancers and several clinical and histolopathological factors are implicated in its prognosis. We recently demonstrated that the overexpression of PAR-3 protein encoded by the *PARD3* gene could be implicated in renal oncogenesis. The object of this work was to study the association of intratumoral PAR-3 expression with known prognostic parameters and clinical outcome.

In this aim, PAR-3 expression was assessed by immunohistochemistry in ccRCC tumors of 101 patients from 2003 to 2005. The immunostaining of PAR-3 was scored either as membranous (mPAR-3) or as both membranous and cytoplasmic (cPAR-3).

Cytoplasmic PAR-3 was significantly associated with worse histopathological and clinical prognostic factors: Fuhrman grades 3 and 4, tumor necrosis, sarcomatoid component, adrenal invasion, renal and hilar fat invasion, eosinophilic component, a non-inactivated *VHL* gene, higher tumor grade, lymph node involvement, metastasis and worse clinical Eastern Cooperative Oncology Group (ECOG) and S classification scores. After multivariate analysis, two parameters were independently associated with cPAR-3: necrosis and eosinophilic components. In addition, cPAR-3 patients had shorter overall and progression free survivals independently from strong prognostic validated factors like metastases.

A cytoplasmic expression of PAR-3 is therefore implicated in worse clinical and pathological cancer features in ccRCC and could be useful to identify patients with high risk tumors.

MAIN TEXT

INTRODUCTION

Renal cell carcinoma (RCC) is by far the most widely spread kidney malignancy, and its most frequent histological sub-type, clear cell renal cell carcinoma (ccRCC), represents about 70% of RCC [1,2]. Several prognostic factors have been described and validated in ccRCC, most of them being histological factors, essentially the Fuhrman nuclear grade, the presence of a sarcomatoid component, tumor necrosis, and invasion of the renal or hilar fat by tumor cells [1,3]. Chromosomal aberrations have also been described; losses of chromosomes 9p and 14 and gains of chromosome 8 are rearrangements associated with worse prognosis [4-6]. More than 90% of sporadic ccRCC have a loss of function of the Von Hippel-Lindau (*VHL*) tumor suppressor gene located on chromosome 3p; and tumors with this gene involvement have in opposite a better outcome [4,7]. When ccRCC is diagnosed at an early stage the optimal treatment of localized tumors is surgical resection by radical or partial nephrectomy [8], however approximately 20% of patients are not diagnosed until their disease is advanced or metastatic and one third of patients will develop metastases after initial surgery [9]. Immunotherapy was the established therapy for patients with metastatic ccRCC [10], and since 2005 the understanding of the underlying molecular mechanisms in the oncogenesis of ccRCC led to the development of drugs that target molecular pathways involved in tumor development such as vascular endothelial growth factor receptor (VEGFR) or mTOR inhibitors [11,12].

Recently, we found a significant 1 Mb gene amplification of the 10p11.21 locus encompassing the partition-defective 3 gene (*PARD3*), in addition to its protein overexpression (PAR-3), in a cell line derived from a ccRCC tumor of worse clinical outcome [13]. PAR-3, belongs to a partition defective complex that controls polarity, which is mainly composed of PAR-3, PAR-6 and atypical protein kinase (aPKC) [14]. In mammalian epithelial cells this complex plays a role in controlling apical-basal polarity, asymmetric cell division, and directional cell migration. It is also believed that PAR proteins

may be involved in multiple aspects of oncogenesis as a relationship exists between polarity dysfunction and cancer progression [14-16].

Defective or overexpressed PAR-3 proteins have been described in other cancers related to tumor development or metastases formation, including breast cancer [17,18], hepatocellular carcinoma[19], or skin tumors [20]. In ccRCC, along with less survival rates, we recently showed that the migration of tumor cells might be promoted by the overexpression of PAR-3 and that the cytoskeleton organization was significantly altered [13]. However, the clinical and pathological significance of PAR-3 have not been elucidated. Therefore we investigated PAR-3 expression in a cohort of 101 ccRCC tumors in relation to the clinicopathological and survival characteristics of respective patients.

MATERIALS AND METHODS

Patients and clinicopathological analysis

101 patients who underwent surgery for clear cell renal cell carcinoma from 2003 to 2005 at our institution were included. The Human Ethics Committee of Rennes University School and Hospital approved this study. The pathology reports, as well as the clinical and follow-up data were retrospectively analyzed. Tumor slides of all patients underwent re-examination by the Department of Pathology. Histological factors were evaluated blindly and independently by 2 pathologists (NRL and JD). Each case was screened for the following pathologic parameters: the Fuhrman nuclear grade, tumor necrosis, sarcomatoid features, adrenal invasion, renal or hilar fat invasion, renal vein invasion, microscopic vascular emboli, and cystic and eosinophilic components.

The clinical data collected was the following: age at diagnosis, sex, side, tumor size, T stage, N stage, M stage, and multifocality. The TNM stage of tumors was upgraded in accordance with the 2009 TNM classification [21]. Two clinical scoring systems, the Eastern Cooperative Oncology Group (ECOG) and the symptom based classification (S classification) were established and used to compare patients' clinical conditions. Patients were stratified in two ECOG groups corresponding to a score of 0 or ≥ 1

[22,23], and two S classification groups S1 (asymptomatic) or S2-S3 (tumors with local or systemic symptoms) [24].

PAR-3 analysis

Immunohistochemistry

For each renal carcinoma case, a representative slide of the tumor with the highest Fuhrman nuclear grade and the corresponding paraffin block were selected. PAR-3 expression was assessed by immunohistochemistry as previously described [13]. The reactivity of antibodies against PAR-3 (dilution 1:50) was revealed with HRP-labeled polymer conjugated secondary antibodies using diaminobenzidine (DAB) as chromogen (Sigma-Aldrich, France). Antibody staining was observed using an Olympus BX51 microscope and images recorded with an Olympus DP70 camera. PAR-3 tumor expression was independently evaluated (NRL), without knowledge of patient outcome. The pattern of PAR-3 staining was qualitatively identified as membranous (mPAR-3) or membranous and cytoplasmic (cPAR-3). Negative control was performed by omitting the primary antibody.

FISH

FISH assays were performed on 16 verified tumor samples of cPAR-3 and m-PAR-3 cases as previously described [25]. The *PARD-3* probe corresponded to a 400 kb BAC contig (RP11-14K21, RP11-981G17, RP11-113B5, RP13-404M3) containing this gene and labeled with Spectrum Green (Abbott, Rungis France). A control probe (RP11-142C8, RP11-106O122, RP11-16P8) was combined, hybridizing at the 10q26.13 locus and labeled with Spectrum Orange (Abbott). All slides were independently analyzed by two observers (FD and FC).

VHL status analysis

VHL gene mutation, deletion and methylation status were assessed as previously described [26]. Each tumor was classified in 2 groups, inactivated *VHL* if a mutation and deletion and/or hypermethylation was detected; otherwise tumors were classified as wild-type *VHL*.

Statistical analysis

All analyses were conducted through procedures available in SAS 9.3 (SAS Institute, Cary N.C., USA). For a descriptive purpose, data was reported as mean and standard deviation or median, 25th and 75th percentiles for continuous variables and follow-up time; numbers and percentages of non-missing values were used for categorical variables. Two-sided p-values from chi-square and t-tests assessed differences between cPAR-3 and mPAR-3 tumors; if needed Fisher exact test and Wilcoxon test were used instead. Maentel-Haenszel chi-square test (Ridit Scores) was used for ordinal variables (the S-System score). A logistic regression model with Firth's bias correction was run in which PAR-3 status (cPAR-3 vs. mPAR-3 tumors) was regressed on the selected covariates, i.e. those covariates significant in the univariate analyses at the $p = 0.15$ level. A backward elimination procedure retained variables with a $p < 0.05$ level. The final model provided estimated probabilities for cPAR-3 according to different pattern of covariates. For overall survival and progression free survival, Kaplan-Meier method calculated product-limit survival estimates according to PAR-3 status and Log-rank test was used to compare survival distributions. Cox proportional hazards modeling was then applied. The multivariable model contained at the outset all covariates significant in the univariate analyses at the $p = 0.15$ level. We used a hand-made stepwise backward elimination process. The proportional hazard assumption was assessed also through graphical (Schoenfeld residuals analysis) as well as testing (using time-dependent variable) approaches.

RESULTS

Patients and tumor characteristics are summarized in Table 1. Our cohort consisted of 58 men (57 %) and 43 women (43 %). Mean age at diagnosis was 62.7 +/- 11.5 years. No difference in age or sex was observed between cPAR-3 and mPAR-3 tumors. The mean duration of follow-up was 59.5 months; the maximal duration was 72 months.

PAR-3 analysis

Immunohistochemistry

Immunohistochemical analysis of healthy kidney tissue showed that PAR-3 was localized at the apical border of tubular cells. A cytoplasmic staining of PAR-3 was observed in 51 out of 101 primary tumors (51.51 %). These cases contained cells with a cytoplasmic, strong and diffuse expression of PAR-3; whereas the immunostaining pattern of mPAR-3 tumors was membranous and diffuse (Figure 1). Considering tumor heterogeneity, 21 cPAR-3 tumors presented different Fuhrman grades on the same slide. In these cases, a cPAR-3 expression was observed in high Fuhrman grades 3 and 4 (including 11 sarcomatoid cases) whereas Fuhrman 2 areas showed only an mPAR-3 staining. In addition, no difference in PAR-3 immunostaining distribution in relation to the vascular supply was observed (data not shown).

FISH

PARD3 gene status was assessed to determine if a cytoplasmic PAR-3 expression was associated to a gene amplification. Interphase FISH analysis was performed on tissue sections of 13 cPAR-3 and 3 mPAR-3 cases. For each tumor, a 2 red-2 green hybridization pattern was detected in at least 70% of the nuclei (Figure 1). Only one tumor showed 3 red and 3 green signals in 10% of the nuclei (data not shown). As a result, no amplification of the *PARD3* gene was detected.

Clinicopathological analysis

Mean tumor size was 7.34 +/- 3.70 cm, with a significant difference between cPAR-3 and mPAR-3 tumors (8.77 +/- 3.85 *vs.* 5.88 +/- 3.01 cm respectively; $p < 10^{-4}$). Most histological prognostic factors were significantly associated to a cytoplasmic expression of PAR-3 (Figure 2): a higher nuclear Fuhrman grade 3 or 4 ($p < 10^{-8}$), the presence of necrosis ($p < 10^{-7}$), eosinophilic ($p < 10^{-10}$) or sarcomatoid features ($p < 10^{-5}$), renal or hilar fat invasion ($p < 10^{-3}$ and $p = 0.033$ respectively), and exclusively the invasion by contiguity of the adrenal gland ($p = 0.013$).

cPAR-3 was associated to poor clinical parameters: higher T stages T3 or T4 ($p < 10^{-3}$) as well as N1/2 and M1 stages ($p < 10^{-3}$ and $p < 5x10^{-3}$ respectively). Tumors with T3/4 stages (48/101 cases) were mainly composed of Fuhrman grades 3 or 4 (43/48). Only 5 cases were of Fuhrman grade 2 and had mPAR-3 stainings. The 11 cases of nodal involvement were only observed in cPAR-3 tumors.

Patients with tumors expressing a cytoplasmic PAR-3 had worse clinical scores: ECOG score ≥ 1 ($p = 0.018$) and S classification scores S2 or S3 ($p < 5x10^{-3}$). In opposite, cPAR-3 was not significantly related to multifocality, cystic component, renal vein invasion or microscopic vascular invasion.

VHL status analysis

Given the frequent *VHL* gene defective status in ccRCC, its inactivation was found in 63 cases corresponding to 28 cPAR-3 (55%) and 35 mPAR-3 (70%) cases. These cases were heterogeneously distributed within the 2 alleles as follows: 71 deletions, 13 promoter methylations, and 52 mutations. Data was not available in 7 cases due to technical failure. PAR-3 was more frequently cytoplasmic in the wild-type *VHL* group than in the inactivated *VHL* group (21/51 (41%) *vs.* 10/50 (20%); $p = 0.048$).

Multivariate and survival analyses

In multivariate logistic regression analysis, necrosis (OR = 5.5; 95%CI, 1.7 to 17; $p = 0.0038$) and the presence of an eosinophilic component (OR = 18; 95%CI, 4.8 to 70; $p < .0001$) were independently associated with cPAR-3 (Figure 2). Kaplan-Meier survival analysis showed a significant difference in both overall ($p < 10^{-4}$) and progression-free survival ($p < 10^{-4}$) between cPAR-3 and mPAR-3 tumors. cPAR-3 tumors had a median overall survival of 28 months and a median progression-free survival of 11 months. In opposite, the median overall and progression-free survivals of mPAR-3 patients were not reached after a 72 month follow-up. At this time point, 64% of mPAR-3 patients were still alive compared to only 31% of cPAR-3 patients. Taking account of the T stage and presence of metastasis, PAR-3 was independently associated with a worse overall survival in multivariate analysis ($p = 0.015$, HR = 2.1, 95% Hazard Ratio Confidence Limits: 1.2 - 3.9). In addition, PAR-3 was an independent

factor for a worse progression-free survival (HR = 2.0; 95%CI, 1.2 to 3.6; p = 0.011) in a multivariate analysis adjusting for vascular emboli, metastasis and the ECOG score. A cytoplasmic PAR-3 expression was also significantly associated with disease-related death (Figure 3).

DISCUSSION

The results of this study underscore that a cytoplasmic PAR-3 expression is a predictor of at-risk tumors, as is supported by worse clinical and histolopathological features and its association to reducing the survival rates of ccRCC patients. To our knowledge, this is the first study which describes PAR-3 expression profile as an independent factor of mortality in renal carcinoma.

Cell polarity is regulated by proteins of the PAR complex which in mammalian epithelial cells play a role in controlling apical-basal polarity, asymmetric cell division, and directional cell migration [14-16]. Mammalian tumors have been reported to aberrantly express PAR-3; its expression is significantly reduced in human invasive breast cancer [17], but is also down regulated in metastasis and associated with a higher tumor grade [18]. In hepatocellular carcinoma, an over-expression of PAR-3 can predict an increased incidence of extra hepatic metastasis and is associated with poor survival [19].

At a genetic level, cell lines and primary tumors of glioblastomas and squamous carcinomas including esophageal, head and neck, and lung squamous cell carcinomas have *PARD3* deletions [27]. Conversely, Zitzelsberger *et al.* reported a *PARD3* amplification using FISH technique in cell lines of radiation-transformed neoplastic retinal pigment cells [28]. This was also observed in a ccRCC cell line of our previous study [13]. Interestingly, FISH analyses performed on 13 cPAR-3 tumors herein demonstrated no gene amplification suggesting that post-transcriptional and/or translational events might be more frequently at the origin of a PAR-3 aberrant expression. The cPAR-3 tumors were related to a non-inactivated *VHL* status, which is associated to a worse prognosis. As *VHL* gene is widely implicated in ccRCC oncogenesis [7], the hypothesis of a possible intracellular interaction between PAR-3 and VHL will be further investigated.

Tumors exhibiting adverse pathological features were significantly associated with a cPAR-3 expression, especially a higher Fuhrman grade and necrosis which are main features of worse outcome in ccRCC [1,3]. Moreover, an eosinophilic component, described to be more frequently observed in high grade ccRCC [29], was also significant in these tumors. The presence of necrosis can be related to a more important tumor proliferation rate. However, the molecular mechanisms associating these major pathological factors to a PAR-3 aberrant expression are unclear and need to be further elucidated. The association between sarcomatoid features and cPAR-3 tumors can be supported by the loss of intercellular adhesions and apico-basal polarity between epithelial cells, which may induce invasive cancers in relation to epithelial to mesenchymal transition [30].

Tumor size was significantly higher in cPAR-3 expressed ccRCCs, and could favor higher probability for extra-renal expansion, especially to the surrounding renal and hilar fat. These factors combined play a role in the T stage status, leading to more frequent T3 or T4 stages. In addition, the N and M stages were significantly higher in cPAR-3 tumors, which is consistent with our previous study suggesting that an over-expression of PAR-3 was associated with tumor cell migration [13]. This finding is also highlighted by a higher frequency of tumor invasion to the perirenal fat as well as to the adrenal gland by contiguity in cPAR-3 ccRCCs.

Two clinical scores were used to evaluate patients at diagnosis [22-24]. The ECOG scale of cPAR-3 patients was significantly higher than that of mPAR-3 patients, meaning that restriction in activity and performance in daily life was more frequent. Similarly, the S classification revealed more frequent symptomatic tumors in cPAR3 patients. Finally, the adjustment of survival rates data to other prognostic factors including metastases reinforced the fact that PAR-3 appears to be a strong independent predictor of poor overall and progression-free survivals.

CONCLUSIONS

Cytoplamic PAR-3 expressed ccRCCs are at significant risk of cancer progression and mortality. Biomarkers introduced into daily practice are essential for a personalized medical care in order to

predict clinical evolution. PAR-3 expression can be easily screened by immunohistochemistry in routine and the assessment of this expression may prove useful to identify high risk ccRCC patients even in absence of usual prognostic parameters. Further investigations need to be undertaken to determine its potential role as a therapy resistance biomarker adding value to existing nomograms.

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TABLE/FIGURE LEGENDS

Table.

Patients and tumor characteristics

Parameters	cPAR-3: n (%)	*P*
Overall tumors (N = 101)	51 (50,5)	
Age at diagnosis, y		.81
<60 (n = 41)	19 (46,3)	
≥60 (n = 60)	32 (53,3)	
Sex		.55
Male (n = 58)	31 (53,4)	
Female (n = 43)	20 (46,5)	
Multifocality		.55
Neg (n = 89)	46 (51,7)	
Pos (n = 12)	5 (41,7)	
Cystic component		.46
Neg (n = 82)	43 (52,4)	
Pos (n = 19)	8 (42,1)	
Fuhrman grade		7.681×10^{-9}*
F 1/2 (n = 32)	3 (9,4)	
F 3/4 (n = 69)	48 (69,6)	
Necrosis		6.863×10^{-8}**
Neg (n = 43)	8 (18,6)	
Pos (n = 57)	42 (73,7)	
NA (n = 1)	1 (100)	
Sarcomatoid differentiation		1.815×10^{-6}*
Neg (n = 74)	26 (35,1)	
Pos (n = 23)	21 (91,3)	
NA (n = 4)	4 (100)	
Eosinophilic component		2.025×10^{-11}**
Neg (n = 38)	3 (7,9)	
Pos (n = 60)	45 (75)	
NA (n = 3)	3 (100)	
Adrenal invasion		.0125*
Neg (n = 93)	43 (46,2)	
Pos (n = 7)	7 (100)	
NA (n = 1)	1 (100)	
Renal fat invasion		2.017×10^{-4}*
Neg (n = 62)	22 (35,5)	
Pos (n = 39)	29 (74,4)	
Hilar fat invasion		.0327 *
Neg (n = 67)	28 (41,8)	

Parameters	cPAR-3: n (%)	*P*
Pos (n = 32)	21 (65,6)	
NA (n = 2)	2 (100)	
Renal vein invasion		.0897
Neg (n = 68)	30 (44,1)	
Pos (n = 33)	21 (63,6)	
Microscopic vascular emboli		.0528
Neg (n = 68)	29 (42,6)	
Pos (n = 32)	21 (65,6)	
Not available (n = 1)	1 (100)	
VHL status		.0478 *
Inactivated (n =63)	28 (44,4)	
Noninactivated (n = 31)	21 (67,7)	
NA (n = 7)	2 (28,6)	
T stage		6.680×10^{-4}*
T1/2 (n = 53)	18 (34)	
T 3/4 (n = 48)	33 (68,8)	
N stage		5.347×10^{-4}*
N0 (n = 90)	40 (44,4)	
N 1/2 (n = 11)	11 (100)	
M stage		.0035*
M 0 (n = 73)	30 (41,1)	
M 1 (n = 28)	21 (75)	
ECOG score		.0182*
0 (n = 69)	29 (42)	
>1 (n = 32)	22 (68,8)	
S-System score		.0018*
1 (n = 47)	15 (31,9)	
2 (n = 26)	16 (61,5)	
3 (n = 28)	20 (71,4)	

Table 1. Patients and Tumor characteristics

Cytoplasmic PAR-3 expression cases compared to the whole group of ccRCC tumors, in relation to clinical and histological data: number of cases and percentages (*: $p < 0,05$, **: independent factor after multivariate analysis).

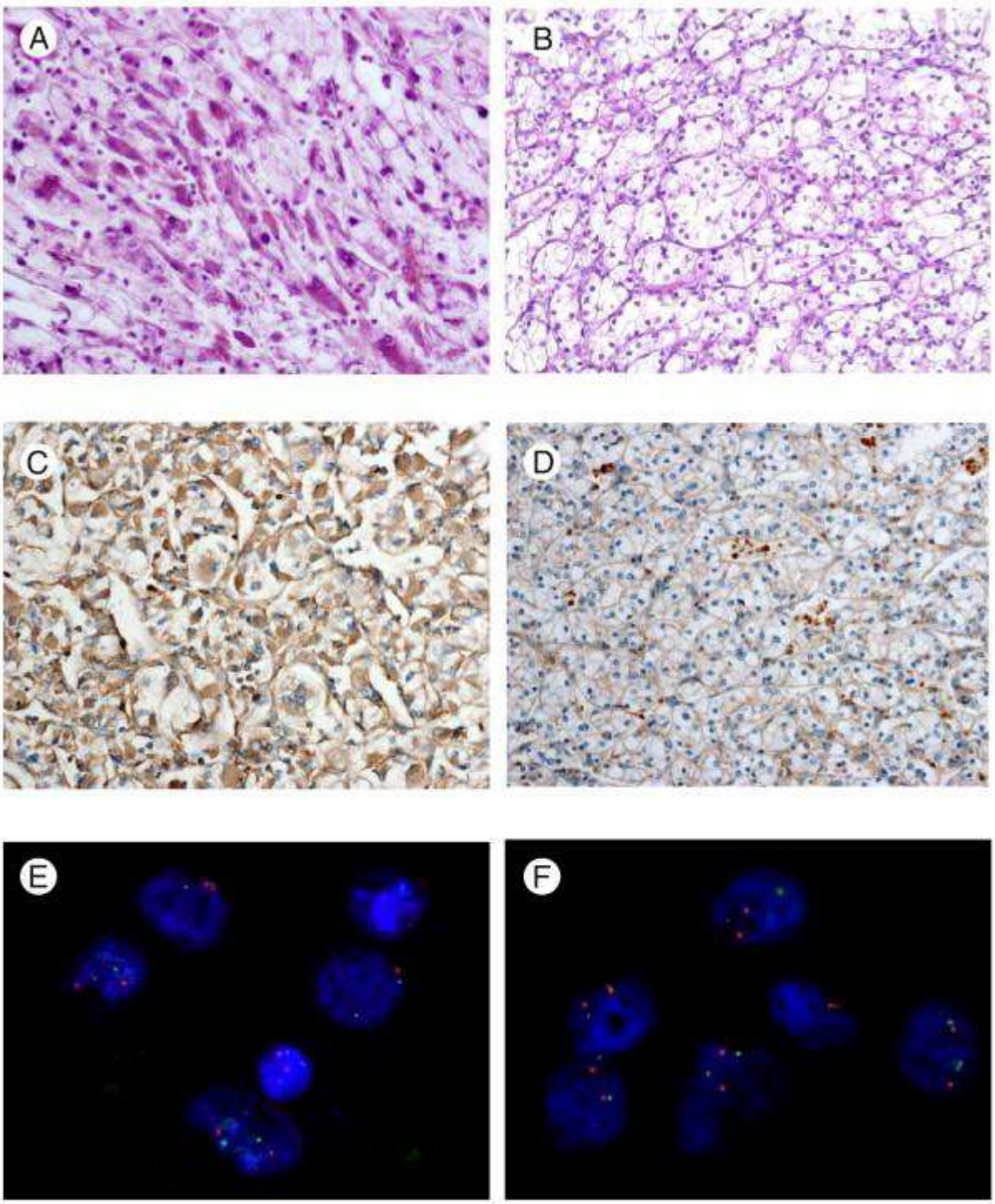

Figure 1. Comparative histological, immunostaining and FISH analysis of distinct cPAR-3 and mPAR-3 cases

cPAR-3 (A, C, E) and mPAR-3 tumors (B, D, F) have different microscopic profiles. (A) sarcomatoid component is associated to necrosis and higher nuclear grade. (B) Classical aspect of a Fuhrman grade 2 ccRCC. (C, D) Immunostaining showing cPAR-3 with immunolabeling located in the cytoplasm and

membrane of tumor cells, while mPAR-3 shows only a membranous staining. (E, F) FISH analysis showing no *PARD3* amplification in both cPAR-3 and mPAR-3 tumors.

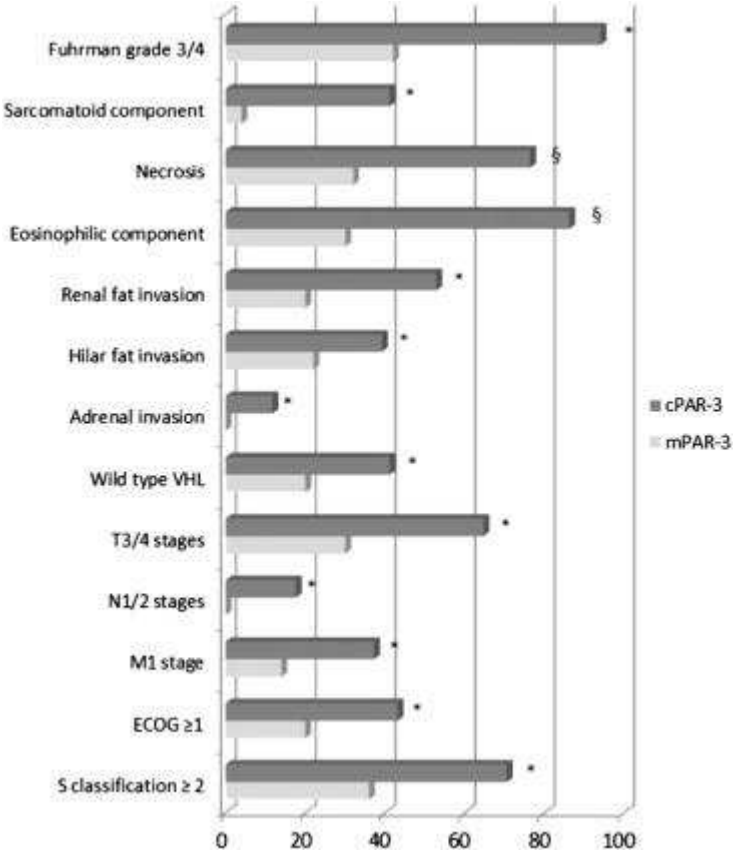

Figure 2. Pathological and clinical features according to PAR-3 expression

Percentage of samples displaying the indicated features within the mPAR-3 and cPAR-3 subgroups. cPAR-3 tumors are significantly associated to poor prognostic factors (*). Necrosis and eosinophilic components remain significant after multivariate analysis (§).

(*: $p < 0.05$; §: significant in multivariate analysis)

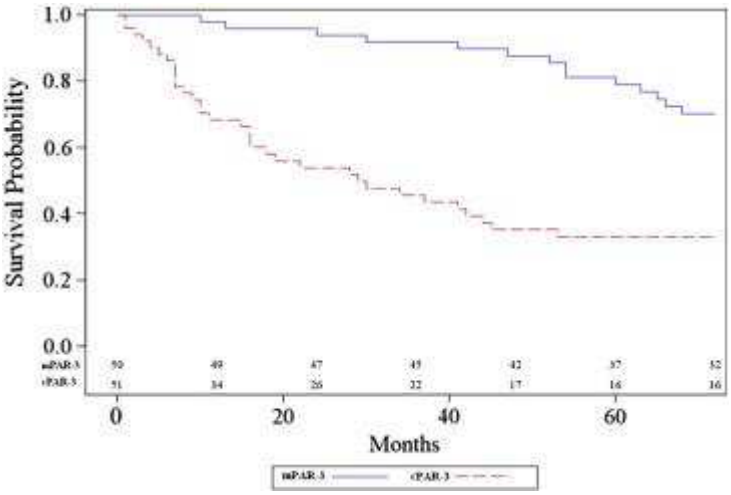

Figure 3. PAR-3 expression and disease-related death

Patients with cPAR-3 tumors have significantly shorter survival than those with tumors having only a membranous expression ($p < 10^{-4}$). Values above the abscissa axis correspond to the number of patients still alive in each group (cPAR-3 and mPAR-3).