Rho GDP dissociation inhibitor-β in renal cell carcinoma

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Abstract. Rho GDP dissociation inhibitor-β (ARHGDIB) is an important mediator of cell signaling. The expression of ARHGDIB is associated with tumor growth and metastasis in a variety of non-genitourinary cancers; however, the role of ARHGDIB in renal cell carcinoma (RCC) has not yet been evaluated. In the present study, tissue samples from 105 patients undergoing surgery for RCC were obtained. The expression levels of ARHGDIB mRNA in normal kidney tissues and in corresponding cancer tissues were analyzed by reverse transcription-quantitative polymerase chain reaction. Differences in relative mRNA expression levels were assessed using paired two-sample t-tests. Expression levels were analyzed with respect to various clinical parameters, and associations were tested using a bivariate logistic regression model. Relative mRNA expression levels in healthy renal tissues compared with cancerous tissues from the same kidney were assessed using paired t-tests. Expression data were compared with respect to survival data by the Kaplan-Meier method/Cox regression analysis. The results revealed that the relative mRNA expression level of ARHGDIB was significantly higher in the lysates of RCC tumor tissues (P<0.001) when compared with healthy renal tissues in a paired analysis of 74 samples; this finding was consistent with the analysis of ARHGDIB mRNA expression levels in all RCC samples, as well as in the subset of clear cell RCC (ccRCC) samples. The relative mRNA expression level of ARHGDIB was also increased in ccRCC tissues compared with papillary RCC tissues (P<0.001). On univariate Cox regression analysis, recurrence-free survival (RFS) was significantly associated with metastasis, locally advanced disease and tumor grade (P=0.018, P=0.002 and P<0.001, respectively). Furthermore, in the subgroup of patients with ccRCC, increased ARHGDIB mRNA expression was significantly associated with a longer RFS time (P=0.001). In summary, the results indicate that ARHGDIB mRNA is highly expressed in RCC tissues in general and is positively associated with RFS in ccRCC. As ARHGDIB has a known effect on angiogenesis and immune modulation, the present study suggests that the functional analysis of ARHGDIB should be performed in the future.

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Introduction

Renal cell carcinoma (RCC) comprises 2-3% of malignant tumors in the Western world (1). It is an increasingly diagnosed malignancy, with an increased number of cases demonstrating a shift towards presenting with smaller renal masses, most likely due to the increasing use of computed tomography and ultrasonography (2). However, ~30% of patients with RCC initially present with metastatic disease, and approximately half of all patients with localized disease will develop metastases over time (3).

A variety of genetic models have been proposed to account for the development of RCC, taking into account certain risk factors, such as cigarette smoking, hypertension and obesity (4). However, despite the genome-wide analyses conducted thus far, the range of molecular mechanisms underlying the development of RCC are not yet fully understood. Familial and sporadic forms of RCC can be associated with certain genetic alterations, the most common of which is the von Hippel-Lindau tumor suppressor gene mutation (5,6).

For advanced or metastatic disease, therapeutic options remain limited. Cytotoxic chemotherapy agents have yielded disappointing results (7). In a previous study, the treatment of advanced and metastasized RCC was attempted with a cytokine-based therapy with interferon-α and high-dose interleukin-2 (IL-2), under the presumption that an immune response could be triggered against RCC cells. However, this therapy offered little benefit with regard to the overall survival times of the patients (8). It is only in the last decade that a broader understanding of RCC tumor biology and the introduction of inhibitors of tyrosine kinase, multikinase and mammalian target of rapamycin (mTOR) have greatly

improved the therapeutic means for metastatic RCC (9). Aside from targeting tumor neoangiogenesis, immunomodulatory checkpoint inhibition was recently introduced as a novel treatment approach (10). Checkpoint inhibition acts via modulation of tumor-infiltrating T lymphocytes and shows promising results as a second-line treatment option (10). These new therapeutic strategies demonstrate that metastatic RCC can no longer be viewed from the perspective of renal tubule cell biology alone, but rather within an integrative model of tumor biology, which includes angiogenesis, interstitial microenvironment and immunological tissue (11).

ARHGDIB belongs to the Rho protein family, whose members are involved in a variety of cellular functions, including proliferation, signaling, secretion, cytoskeletal organization and proliferation; while also belonging to Ras-associated small GTP-binding proteins (12). Since ARHGDIB is involved in neoangiogenesis and lymphocyte function (two key factors in the treatment of metastasized RCC), it is an area of focus for the study of RCC, and metastatic RCC in particular (13). However, despite serving a role in other malignancies such bladder (14,15), the role of ARHGDIB for RCC has not been evaluated yet. The aim of the present study was to evaluate the role of ARHGDIB in RCC by comparing ARHGDIB mRNA expression levels in healthy and tumor tissue as well as by assessing the potential impact of expression levels of ARHGDIB on survival.

Materials and methods

Tissue sampling. Renal tumor tissues and adjacent corresponding tumor-free renal tissues of 105 patients undergoing kidney surgery for RCC were collected between January 2001 and December 2005. Tissue samples were obtained during surgery. All samples were collected at Eberhard Karls University of Tübingen (Tübingen, Germany) and samples where lymphatic tissue invasion exceeded 25% of the specimen section were excluded. Corresponding adjacent normal tissue was sampled 0.5-2 cm from the surgical margin of the primary tumor and each tumor and normal tissue sample was immediately snap frozen in liquid nitrogen and stored at -80˚C. Tumor stage and histological subtype were assessed according to the 2002 Union for International Cancer Control tumor-node-metastasis system (16) by two independent pathologists that were blinded to mRNA expression of ARHGDIB as well as the clinical course of the patient. Tumor grade was defined in accordance with the Fuhrman grading system (17), while histological subtypes were based on the consensus classification of renal cell neoplasia (18). Localized RCC was defined as pT≤2 without organ metastasis or lymph node involvement and a grade of ≤2. Advanced RCC was defined as pT≥3 or organ metastasis or >G2 or lymph node-positive disease. Only tissue samples that included ≥75% vital tumor tissue were selected. None of the patients received neoadjuvant treatment prior to definitive surgery; thus, all patients in the present study were systemic therapy-naïve. Data were gathered by physicians and passed to data managers for evaluation and storage in a relational database. Written informed consent was obtained from all patients prior to surgery and tissue sampling. Approval was granted prior to the study by the Ethics Committee of the Medical Faculty of Eberhard Karls University.

Patients. The histopathological and clinical characteristics of the patients are summarized in Table I. The mean age of all patients was 63.6 (±11.8) years. The male:female ratio was 67:38 (~1.76:1; 63.8% males and 36.2% females). Histopathological subclassification showed 77 patients with ccRCC, 21 patients with papillary RCC (papRCC) and 5 patients with chromophobe tumors, while 2 patients had non-classified histology.

Primary cells. Renal proximal tubular epithelial cells (RPTEC; Lonza Group, Ltd., Basel, Switzerland) were cultured and prepared according to the manufacturer's protocol as an external reference control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissue was prepared from 20 cryosections of 20-μm thickness. Using TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), total RNA was extracted. Two sections of each tissue sample were stained with hematoxylin-eosin and evaluated by a pathologist. The conversion of RNA into single-stranded complementary DNA (cDNA) was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The TaqMan assays used were as follows: ARHGDIB (assay ID: Hs00171288_m1), RPL13A (assay ID: Hs0-304-3885_g1), HPRT1 (assay ID: Hs9-999-9909_m1) and GUSB (assay ID: Hs0-093-9627_m1). For RT-qPCR, the ABI 7900 Fast Sequence Detection System with Universal PCR Master Mix and TaqMan Expression Assays (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used exactly as described in the manufacturer's TaqMan Gene expression assay protocol PN 4333458N: Standard 40 cycles of denaturation at 95˚C (15 sec) and annealing and extension at 60˚C (60 sec) were applied for amplification of cDNA, after standard initial denaturation and TaqPolymerase enzyme activation at 95˚C (10 min). All evaluations were duplicates meaning that each cDNA has been analyzed twice and mean values were used for further evaluations. For the biological control, cDNA generated from RPTEC primary cells was used (50 ng cDNA in 1 μl per reaction). For endogenous controls, transcripts of the human RPL13A, HPRT1 and GUSB were used; these were combined using the dataAssist software (version 2.0; Thermo Fisher Scientific Inc.) and the arithmetic mean was used as a method for normalization. Blank no-template and no RT controls were inserted for each measurement. The method of Livak and Schmittgen (19) and reference ΔCq values originating from the biological reference RPTEC were used for the calculation of ΔΔCq and all relative quantity values. In total, 105 measurements for ARHGDIB expression were successfully performed.

Statistical analysis. Data were assessed using the SDS 2.3 Manager, dataAssist software (version 2.0; Applied Biosystems; Thermo Fisher Scientific Inc.). Statistical programming language R 2.15-2 (R core team; R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical calculations. The two-sided type-I error was set to 5% for all statistical tests. All graphical plots were generated using R statistical software (20). For statistical analysis of ARHGDIB mRNA expression in tumor vs. paired adjacent tissue, the Wilcoxon signed-rank test and paired t-test were

Table I. Histopathological and clinical parameters of patients with renal cell carcinoma.

Characteristics	Value
Total, n	105
Mean age ± standard deviation, years	63.6±11.8
Sex, n (%)	
Male	67 (63.8)
Female	38 (36.2)
Median tumor diameter, cm	4.5
Histology, n (%)	
Clear cell	77 (73.3)
Papillary	21 (20.0)
Chromophobe	5 (4.8)
Other/not classified	2 (1.9)
Stage, n (%)	
Not applicable	3 (2.9)
pT1	9 (8.6)
pT1a	32 (30.5)
pT1b	20 (19.0)
pT2	4 (3.8)
pT3	3 (2.9)
pT3a	10 (9.5)
pT3b	24 (22.9)
pT4	0 (0.0)
Grade, n (%)	
G1	17 (16.2)
G1-2	14 (13.3)
G2	57 (54.3)
G2-3	7 (6.7)
G3	10 (9.5)
Lymph node metastasis[a], n (%)	11 (10.5)
Visceral metastasis[a], n (%)	23 (21.9)
Advanced/metastatic disease[b], n (%)	29 (27.6)

[a]At time of surgical intervention. [b]Advanced RCC was defined as pT3-4 or positive lymph node metastasis or positive organ metastasis or grade >2.

Table II. Histopathological and clinical parameters of the patients included in the paired analysis of Rho GDP dissociation inhibitor-β mRNA expression in tumors vs. adjacent normal kidney tissues.

Characteristics	Value
Total, n	74
Mean age ± standard deviation, years	66.5±11.8
Sex, n (%)	
Male	45 (60.8)
Female	29 (39.2)
Histology	
Clear cell	58 (78.4)
Papillary	11 (14.9)
Chromophobe	4 (5.4)
Other/not classified	1 (1.4)
Stage, n (%)	
Not applicable	3 (4.1)
pT1	4 (5.4)
pT1a	24 (32.4)
pT1b	14 (18.9)
pT2	3 (4.1)
pT3	1 (1.4)
pT3a	6 (8.1)
pT3b	19 (25.7)
pT4	0 (0.0)
Grade	
G1	9 (12.2)
G1-2	10 (13.5)
G2	40 (54.1)
G2-3	6 (8.1)
G3	9 (12.2)
Lymph node metastasis[a], n (%)	7 (9.5)
Visceral metastasis[a], n (%)	19 (25.7)
Locally advanced/metastatic disease[b], n (%)	23 (31.1)

[a]At time of surgical intervention. [b]Advanced RCC was defined as pT3-4 or positive lymph node metastasis or positive organ metastasis or grade >2.

used. Logistic regression was used to assess the associations between ARHGDIB mRNA expression and clinical parameters. Kaplan-Meier and Cox's regression analyses were used to determine the effects of mRNA expression patterns on RFS. Data on ARHGDIB for RCC were downloaded from the Kidney Renal Clear Cell Carcinoma (KIRC)/The Cancer Genome Atlas (TCGA) data portal (cancergenome.nih.gov, accessed 4/2014). The data were evaluated with a paired two-sided t-test for paired samples and logistic regression for group comparison.

Results

Comparison of normal vs. paired tumor tissue. Tumor kidney tissue was compared with adjacent, histologically normal-appearing tissue in a subset of 74 paired samples (samples in which paired tissue samples were available). The mRNA expression levels of ARHGDIB were found to be significantly higher in the tumor tissue compared with normal tissue (P<0.001). Subsequently, a paired analysis was performed with the 74 samples. The characteristics of the patients included in the paired analysis are shown in Table II. Significantly increased mRNA expression levels for ARHGDIB were observed for all RCC tissues combined (P<0.001; Fig. 1), and also for the subgroup of ccRCC tissues (P<0.001; Fig. 2). The mRNA expression levels of ARHGDIB were also more pronounced in ccRCC tissues when compared with tissues from papRCC [P<0.001; odds ratio (OR), 0.228 (95% confidence interval, 0.118-0.444)]. The histopathological

Table III. Histopathological parameters and relative expression levels of ARHGDIB mRNA in all patients with RCC.

Sample/pathology	Patients, n	Mean relative ARHGDIB expression (ΔΔCq)	Standard deviation
Total	105		
Sex			
Male	67	-0.43	1.04
Female	38	-0.16	0.76
Age			
Below median (65 years)	54	-0.34	0.98
Equal to or above median (65 years)	51	-0.32	0.93
Histology			
Papillary RCC	28	-1.29	0.97
Clear cell RCC	84	-0.05	0.75
M stage			
M0	82	-0.39	1.04
M+	23	-0.12	0.48
N stage			
N0	94	-0.30	0.96
N+	11	-0.56	0.85
Disease progression[a]			
Localized RCC	76	-0.36	1.04
Advanced RCC	29	-0.24	0.68
Grade			
≤2	88	-0.30	0.91
>2	17	-0.52	1.15

[a]Advanced RCC was defined as pT3-4 or N+ or M+ or grade >2. ARHGDIB, Rho GDP dissociation inhibitor-β; RCC, renal cell carcinoma; N+, positive lymph node metastasis; M+, positive organ metastasis.

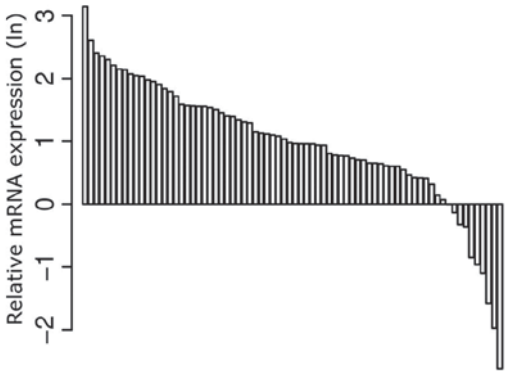

Figure 1. Assorted differences plot for paired analysis showing the highly significant difference between relative logarithmic (ln) expression of Rho GDP dissociation inhibitor-β mRNA in RCC tissue compared with the surrounding normal kidney tissue (P<0.001) in all RCC samples. RCC, renal cell carcinoma.

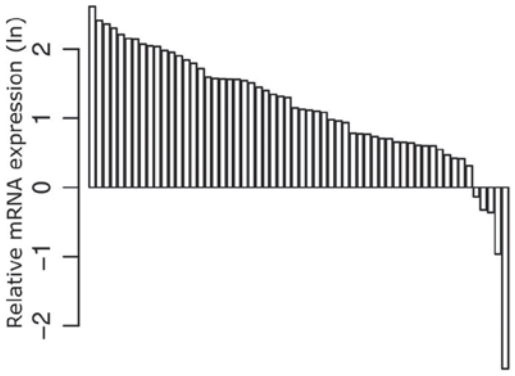

Figure 2. Assorted differences plot for paired analysis demonstrating the difference between relative logarithmic (ln) expression of Rho GDP dissociation inhibitor-β mRNA in clear cell renal cell carcinoma tissue compared with the surrounding normal kidney tissue (P<0.001).

and clinical parameters and relative expression levels (ΔΔCq) of ARHGDIB mRNA are summarized in Table III.

Recurrence-free survival. When analyzing clinicopathological parameters in univariate Cox regression analysis, RFS was significantly associated with the occurrence of metastases, locally advanced disease and tumor grade (P=0.018, P=0.002 and P<0.001, respectively). For RCC, including all pathological subtypes, bivariate Cox regression did not show any association of ARHGDIB mRNA expression with RFS (Table IV).

For the subgroup of ccRCC, higher expression levels (threshold-0.45 ΔΔCq) of ARHGDIB were associated with longer RFS time on univariate Cox regression analysis [hazard ratio, 0.11; 95% confidence interval (CI), 0.03-0.46; P=0.002].

Table IV. Cox's regression analyses of clinical parameters for recurrence-free survival.

Variable	Hazard ratio	95% confidence interval	P-value
Metastasis (RCC)	5.71	1.91-17.1	0.018
Locally advanced disease (RCC)	5.47	1.83-16.4	0.002
Tumor grade (RCC)	12.7	3.89-41.3	<0.001
ARHGDIB mRNA expression (ccRCC)	0.11	0.03-0.46	0.002

RCC, renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; ARHGDIB, Rho GDP dissociation inhibitor-β.

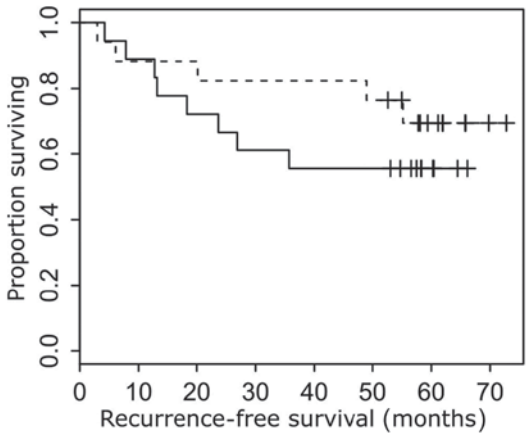

Figure 3. Kaplan-Meier analysis of recurrence-free survival according to ARHGDIB expression in clear cell renal cell carcinoma tissue. Higher ARHGDIB expression was associated with a longer RFS (hazard ratio, 0.11; 95% confidence interval, 0.03-0.46; P=0.0024) on univariate Cox regression analysis. ARHGDIB, Rho GDP dissociation inhibitor-β.

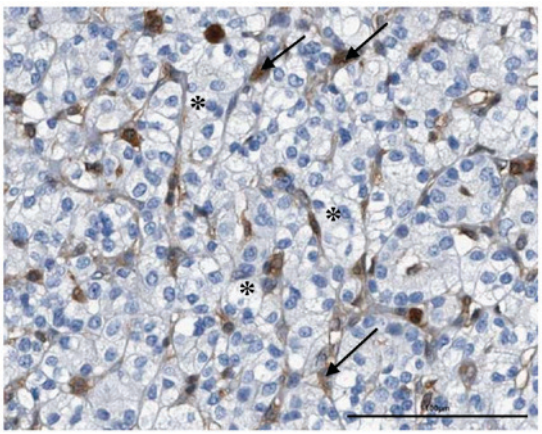

Figure 4. Immunostaining and hematoxylin counterstain of Rho GDP dissociation inhibitor-β in interstitial tissue revealed that lymphocytes (arrows) exhibited positive staining, in contrast to ccRCC cells (*). Image credit: The Human Protein Atlas (45,46). Scale bar, 100 μm.

A bivariate Cox regression model, adjusted for metastatic status (P=0.001), tumor diameter (P=0.043), advanced disease (P=0.030) and lymph node metastasis (P=0.006) was also able to identify increased ARHGDIB mRNA expression as a positive prognostic factor for RFS in patients with ccRCC histology (Fig. 3).

Comparison with TCGA data. Upon interrogation of the TCGA/KIRC database (http://cancergenome.nih.gov/) for 479 tested tissue samples, similar results were observed. ARHGDIB mRNA expression was associated with RCC (P<0.001), but not with the presence of metastasis (P=0.586; OR, 1.11), positive lymph nodes (P=0.141; OR, 1.22), tumor stage (P=0.053; OR, 1.32) or grade (P=0.463, OR, 1.11).

Discussion

The majority of members of the Ras family of GTPases act as molecular regulators, switching between an active GTP-bound state with protein localization at the cellular membrane, and the cytosolic inactive protein bound to GDP (21). Rho GTPases can be regulated by GDP dissociation inhibitors, including ARHGDIB (22). There is evidence that ARHGDIB is expressed differently in different tumor entities at the protein and mRNA levels; for example, ARHGDIB mRNA expression has been found to be elevated in adenocarcinoma of the ovaries (23) and shown to be positively correlated with tumor progression in gastric cancer (24). Furthermore, increased mRNA expression has been detected in breast cancer, and demonstrated to be associated with increased cell motility and invasiveness in *in vitro* experiments (25). By contrast, low ARHGDIB expression has been shown to be associated with the invasive capacity and clinical prognosis of bladder cancer (14,15).

The two major therapeutic approaches to treatment of metastasized or inoperable advanced RCC have been immunotherapy via interferon-α and IL-2 (8), as well as targeted antiangiogenic therapy more recently (10). ARHGDIB is involved in carcinogenesis in a variety of malignancies, and serves roles in neoangiogenesis and signaling in the lymphocyte immune response (12-15), which makes it an interesting target for investigation with regard to metastatic RCC.

Neoangiogenesis is associated with hypoxia-inducible factor (HIF) transcription factors, which perform critical roles in cell metabolism with regard to oxygen homeostasis (26). HIF-α accumulates under hypoxic conditions and is degraded in the presence of oxygen (26). HIF accumulation results in the overexpression of a variety of growth factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which promote neoangiogenesis (27). It is well known that the type and density of vasculature in RCC are associated with tumor histological grade and extent of macroscopic tumor necrosis, which affect the clinical course of RCC (28), including the ccRCC subgroup (29).

Increased understanding of the molecular aspects of angiogenesis in tumor tissues has led to the development of targeted therapy for RCC (30), which is a highly-vascularized tumor entity (29). Sunitinib, one of the first novel therapeutic agents to be used for RCC, modulates the VEGF-C pathway (31). It is a tyrosine kinase inhibitor that acts against VEGF receptors 1, 2 and 3, as well as PDGF receptors α and β (32,33). In response to therapy with sunitinib, the plasma levels of VEGF-C decrease and patients show a markedly improved RFS (31). Bevacizumab, another therapeutic agent for the treatment of metastasized RCC, is an antibody that targets VEGF, and thereby also acts against tumor neoangiogenesis via VEGF inactivation, leading to an improved RFS time for patients with ccRCC (34).

Recently, a functional association between ARHGDIB and VEGF-C was detected for gastric cancer; ARHGDIB overexpression was demonstrated to lead to elevated mRNA and protein levels of VEGF-C (13). By contrast, a decreased level of ARHGDIB in the gastric cancer MKN-28 cell line was associated with markedly decreased VEGF-C expression (13). Therefore, it has been demonstrated that VEGF-C is among the mediators that induce angiogenesis *in vivo* (35), and that it also promotes cancer cell invasion and metastasis (36,37). These results, which functionally link VEGF-C and ARHGDIB, indicate a possible involvement of ARHGDIB in neoangiogenesis and carcinogenesis.

Another possible functional association between ARHGDIB and RCC signaling is the potential connection of ARHGDIB to the invasion of lymphocytes into tumor tissues. It has previously been shown that ARHGDIB is strongly expressed in lymphatic tissue (38), and that RCC tumors frequently exhibit varying amounts of infiltrating lymphocytes (39). The interaction between RCC and the lymphocyte-containing microenvironment is of special importance, as almost all RCCs appear to express cluster of differentiation 70, which drives proliferative exhaustion of lymphocytes and may be a possible mechanism underlying the immune evasion of the tumor (40).

A previous study observed the overexpression of ARHGDIB in human monocytes, leading to the subsequent suppression of Rac membrane localization, disrupting the actin cytoskeleton and thereby negatively effecting phagocytosis (41). Groysman *et al* (42) were able to demonstrate that ARHGDIB, a member of the Rho GTPases, is also pivotal for T cell signaling, where it is involved in the regulation of intracellular signaling pathways leading to nuclear factor of activated T cells stimulation, which in turn leads to the biosynthesis of IL-2 and subsequent activation and proliferation of T lymphocytes. This association with lymphocyte metabolism poses a possible association with the previously mentioned immune-based therapeutic approaches for RCC with IL-2 (43), which were used prior to the advent of tyrosine kinase inhibitors.

T cell signaling is also the mechanism by which certain novel pharmacological agents exert their antitumor effect. Checkpoint inhibitors have shown promising results in urological and non-urological cancers; recently, the targeting of programmed death receptor on T cells has shown superiority in comparison with the mTOR inhibitor everolimus in the treatment of RCC (10,44). This has led to the implementation of the checkpoint inhibitor nivolumab as a second-line treatment option for metastatic RCC.

One limitation of the present study was the varying amounts of lymphatic cell invasion, vasculature and interstitial tissue among the included cases. The protein expression of ARHGDIB in normal and tumor renal tissues has thus far only been immunohistochemically analyzed for a small number of tissues, showing more prominent immunopositivity in renal glomeruli, with prominent reactivity in the surrounding lymphatic cells and fibroblasts, but a low or no detectable reactivity in renal tubules (Fig. 4) (45,46). Considering that mRNA expression levels have been measured in tissue lysates following the homogenization of the cells, the present study cannot provide information regarding the origin of the ARHGDIB expression and, furthermore, the results may also show bias due to variations in lymphocyte content in the tissue samples. Lymphocytes are often found infiltrating or surrounding RCC tissues (47). After reviewing the histopathological control sections in the present study, lymphatic tissue invasion that exceeded 25% of the specimen section was excluded. The positive correlation of ARHGDIB with RFS in the subgroup of RCCs with clear cell histology in the present study cannot be explained at present. However, future functional analyses should assess the effects of ARHGDIB on the tumor-surrounding tissues, and particularly lymphocyte function, as this may serve a role.

In conclusion, ARHGDIB mRNA expression is highly upregulated in RCC tissues compared with adjacent normal tissues, and is positively associated with RFS in ccRCC. At present, the two major modes of action for the pharmacological treatment of RCC are angiogenesis inhibition and T-cell checkpoint inhibition. Therefore, the effect of ARHGDIB on angiogenesis, as well as its roles in the cell signaling of lymphatic tissue and immune therapy, makes it an interesting target for further functional studies.

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