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Original Article

ADP-ribosylation factor-like 4C is a predictive biomarker of poor prognosis in patients with renal cell carcinoma

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Abstract: Renal cell carcinoma (RCC) has the high mortality rate among urological malignancies. The development of RCC cannot be effectively reduced by molecular targeted therapies based on nutrient deprivation, such as inhibition of tumor angiogenesis. The objective of this study was to identify predictive biomarkers of poor prognosis and therapeutic molecular targets in patients with RCC. Two independent cohorts were analyzed in the present study. Global transcriptomics were used in the first cohort (43 patients with RCC) to identify biomarker genes. Each identified biomarker was subsequently analyzed using immunohistochemistry in the second cohort (97 patients with RCC). Following transcriptomics, biomarkers were evaluated using receiver operating characteristic curve analysis. Predictive accuracy for poor survivals was assessed using the log-rank test and Cox multivariate analysis. Global transcriptomic analysis in the first cohort focusing on cases with survival periods <2 years after initial diagnosis of metastasis detected seven overexpressed genes, which correlated with poor prognosis. The *ADP-ribosylation factor-like 4C (ARL4C)* exhibited the best accuracy in the receiver operating characteristic curve analysis and predicted poor survival in the first cohort (log-rank test, P<0.001; Cox multivariate analysis, hazard ratio =167, P=0.005). In the second cohort, the expression of ARL4C was semi-quantitatively evaluated through immunohistochemistry. Twenty-seven cases showed high levels of ARL4C, confirming a significant association with shorter survivals (log-rank test, P<0.001; Cox multivariate analysis, hazard ratio =9.41, P=0.004). ARL4C was shown to be a predictive biomarker for poor prognosis in patients with RCC and may be a novel target in the treatment of RCC.

Keywords: ADP-ribosylation factor-like 4C (ARL4C), poor prognosis, predictive biomarkers, renal cell carcinoma (RCC), primary tissues, metastasis, predictive accuracy, global transcriptome, next generation sequencer (NGS), immunohistochemistry

Introduction

Renal cell carcinoma (RCC) has the high mortality rate among urological malignancies and an increasing incidence [1]. Radical nephrectomy remains the standard and only curative therapy for patients with localized RCC. However, one third of new cases are diagnosed with regionally advanced disease or eventually develop distant metastases. Moreover, up to half of the remainder eventually develop distant metastases after the initial treatment [2]. Currently, molecular targeted therapies used in the treatment of patients with advanced RCC include the following two major subgroups of agents: tyrosine kinase inhibitors (e.g., Sorafenib [Nexavar, Bayer], Sunitinib [Sutent, Pfizer], Pazopanib [Votrient, GlaxoSmithKline], and Axitinib [Inlyta, Pfizer]) [3-7], and specific inhibitors of the mammalian target of rapamycin (mTOR) kinase (e.g., Temsirolimus [Torisel, Pfizer] and Everolimus [Afinitor, Novartis]) [8, 9]. The common rationale for use of these agents to suppress the development of cancer is based on nutrient deprivation, including inhibition of tumor angiogenesis. However, the therapeutic efficacies of these agents are not sufficient. In the last decades, the treatment of advanced RCC has evolved dramatically due to the introduction of targeted therapies and novel immunotherapies. The CheckMate-214 trial showed a survival superiority of combined immunother-

apy over targeted therapy in the cohort of patients at intermediate or poor-risk [10]. However, targeted therapies such as tyrosine kinase inhibitors or inhibitors of mTOR may be beneficial and preferred for some patients, even when immunotherapy with checkpoint inhibitors has become a major modality. At this moment, the selection of optimal candidates for targeted agents remains an open question. Biomarkers that can potentially predict patients most likely to respond to targeted treatments are needed.

In a previous study, we demonstrated the presence of two types of cells in RCC for carbon metabolism and for cell signaling under glucose starvation, which is the major nutrient denied to cells following inhibition of angiogenesis [11]. These findings suggested that differences between starvation-resistant and -sensitive RCC cells might be key factors in developing novel targeted therapies. Starvation-resistant cells are considered to be dormant-state cells able to survive even under glucose starvation [11]. Using these two types of RCC cells, we showed that mitochondrial manganese-dependent superoxide dismutase (SOD2) [12] and tumor necrosis factor (TNF)-related apoptosis-induced ligand (*TNFSF10/*TRAIL) [13] were potential markers of poor prognosis. In addition, buformin (a biguanide) [11, 12], etomoxir (an inhibitor of beta-oxidation from fatty acids) [12], and chetomin (a nuclear inhibitor of hypoxia inducible factor [HIF]) [14], may be potential therapeutic agents.

In the present study, we investigated more useful biomarkers, which may predict poor prognosis in patients with RCCs and become therapeutic targets. For this purpose, we used clinically resected RCC tissue samples and performed a global transcriptomic analysis. Our investigation showed that ARL4C was a useful predictor of poor prognosis in patients with RCC through the global transcriptomic and clinicopathological analyses in two independently clinical cohorts; and could become a novel therapeutic target for RCC.

Materials and methods

Patients

In the first cohort, the medical records of patients with RCC treated in the Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan between 2008 and 2014 were retrospectively reviewed. In all patients, the presence of clear cell RCC was pathologically confirmed. The histopathological analysis was performed according to the 2004 World Health Organization classification [15]. This study was conducted in compliance with the declaration of Helsinki and approved by the institutional review board of the Cancer Institute Hospital (2009-1029). All patients provided written informed consent prior to their participation in this study.

In the second cohort, the medical records of patients with RCC treated in the Shiga University of Medical Science Hospital, Shiga, Japan from 1999 to 2016 were reviewed. According to the 2004 World Health Organization classification [15], the diagnosed pathological types of RCC included 76 clear cell cases, 12 papillary cases, eight chromophobe carcinomas cases, and one unclassified case. Clinicopathological data were obtained from medical records following written informed consent from individual patients and approval by the Ethics Committee of the university (No. 27-192).

RNA preparation

Total RNA was extracted from primary tissues of patients with RCC using the Trizol Plus RNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA was quantified using a Bioanalyzer (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. The RNA Integrity Numbers of all prepared total RNA samples were ≥8.

High-throughput DNA sequencing

The library of template molecules for high-throughput DNA sequencing was converted from the total RNA using the TruSeq RNA Sample Prep Kitv2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The library was quantified using a Bioanalyzer (Agilent) following the manufacturer's instructions. The library (4 pM) was subjected to cluster generation on a Single Read Flow Cell v4 (TruSeq SR Cluster Kit v2-cBot-GA) with a cBot generation instrument (Illumina). Sequencing was performed on a Genome Analyzer GAIIx for 58 cycles using Cycle Sequencing v5-GA reagents (Illumina).

Data analysis

Image analysis and base calling were performed using the Real Time Analysis version 1.13 (Illumina). The sequence libraries for each sample were processed using the CASAVA Software 1.8.2 (Illumina) to produce 51-bp sequence data in fastq format. The fastq files were processed using the Cutadapt version 1.2.1 [16] with option -q 30. In addition, we removed reads shorter than 49-bp using Cutadapt. Trimmed reads for each sample were aligned to the reference genome (Ensembl buid GRCh37) using TopHat version 2.0.10 [17] with default setting, except for option -G. The differential gene expression analysis was performed using Cufflinks [18] with option -g and focused on the contrast of three RCC groups. Cuffmerge was used to merge the assembled transcripts into a consensus gene track from all mapped samples with options -s and -g. Moreover, Cuffqunt was used for quantification using option -M, and for the removal of mitochondria genes, immunoglobulins, and human leukocyte antigens. Cuffdiff was used to identify differentially expressed genes and transcripts between these three RCC groups. Genes and transcripts were identified as being significantly differentially expressed with q values ≤0.05, calculated using the Benjamin-Hochberg FDR correction [18]. In addition, the values of fragments per kilobase of exon per million fragments mapped (FPKM) were converted from count values for the comparison of expression levels among genes. All new data were deposited in the DNA Data Bank of Japan (DDBJ) Japanese Genotype-phenotype Archive for genetic and phenotypic human data under accession number JGAS00000000149.

Immunohistochemistry

Surgical specimens were transferred to 10% buffered formalin and fixed overnight. The fixed samples were embedded in paraffin and serially sliced into 5-μm sections. After dewaxing, the sections were autoclaved at 120°C for 1 min in 10 mM sodium citrate buffer (pH 6.0) and immersed in 0.3% H_2O_2. Subsequently, they were incubated overnight at 4°C with primary antibodies against ARL4C (diluted 1:400, #10202-1-AP, Proteintech, Rosemont, IL, USA). The sections were rinsed with phosphate-buffered saline and incubated with a horseradish peroxidase-conjugated secondary antibody (SimpleStain MAX-PO; Nichirei, Tokyo, Japan) for 1 h at room temperature. The sections were subsequently stained with 3.3'-diaminobenzidine tetrahydrochloride and counter-stained with hematoxylin.

Microscopic evaluation for ARL4C immunohistochemistry

ARL4C is expressed in normal proximal renal tubules. Thus, its expression can be used as an internal control for each histological section. Subsequently, the expression of ARL4C in tumors was semi-quantitatively evaluated through relative comparison with those observed in normal proximal renal tubules on the same slide. We defined two qualitative grades of specimens according to the intensity level of ARL4C expression: i) "high ARL4C" samples, in which the intensity of staining in the tumor was equal to or stronger than that observed in normal proximal renal tubules and ii) "low ARL4C" samples, in which the intensity staining in the tumor was lower than that reported in the proximal tubules.

Receiver operating characteristic (ROC) analysis

ROC curve analysis was performed using the ROCR package of the free software R (https://www.r-project.org/). The 95% confidence interval for area under the curve (AUC) was calculated according to Hanley and McNeil (1982) [19].

Statistical analysis

The data were reported as means ± standard error (SE). The values were derived from at least triplicate experiments. Statistical analyses were performed using R. One-way factorial analysis of variance (ANOVA), accompanied by pair-wise comparisons using t tests with pooled standard deviation (SD) was used to compare the means of multiple groups. A $P<0.05$ denoted statistical significance.

The disease-specific overall survival (OS) interval was defined as the period from the point of diagnosis to the point of death caused by RCC. All statistical analyses for clinical evaluation were performed using the SPSS 22.0 (IBM Inc., Chicago, IL, USA). Tests for statistical signifi-

Table 1. The list of genes which showed >90% accuracy in the receiver operating characteristic (ROC) curve analysis, which predicted poor prognosis in renal cell carcinoma (RCC), using FPKM transcriptional values

	Cutoff	TP	FP	FN	TN	Sensitivity	Specificity	Accuracy	AUC (95% CI)
ARL4C	70.63	8	0	1	34	0.88889	1.00000	0.97674	0.9183 (0.7829-1)
BUB1	12.32	6	0	3	34	0.66667	1.00000	0.93023	0.9346 (0.7940-1)
BIRC5	5.93	6	1	3	33	0.66667	0.97059	0.90698	0.8300 (0.6347-1)
CPS1	18.11	5	0	4	34	0.55556	1.00000	0.90698	0.8922 (0.7007-1)
	14.59	6	1	3	33	0.66667	0.97158		
ECT2	18.36	6	1	3	33	0.66667	0.97059	0.90698	0.9052 (0.7512-1)
FSTL1	215.37	6	1	3	33	0.66667	0.97059	0.90698	0.8072 (0.6028-1)
STEAP3	16.99	6	1	3	33	0.66667	0.97059	0.90698	0.8399 (0.6488-1)

ROC analysis was performed using 43 primary RCC tissues containing nine patients with poor prognosis (i.e., survival ≤2 years after initial diagnosis of metastasis) and 34 patients with good prognosis (i.e., survival ≥5 years without or ≥4 years with metastases). The FPKM transcriptional values of genes were significantly higher in the group with poor prognoses versus the group with good prognosis. TP: true positive, FP: false positive, FN: false negative, TN: true negative, Sensitivity, Specificity, and Accuracy: prediction of patient mortality caused by RCC within 2 years after initial diagnosis of metastasis, AUC (95% CI): area under the curve (95% confidence interval).

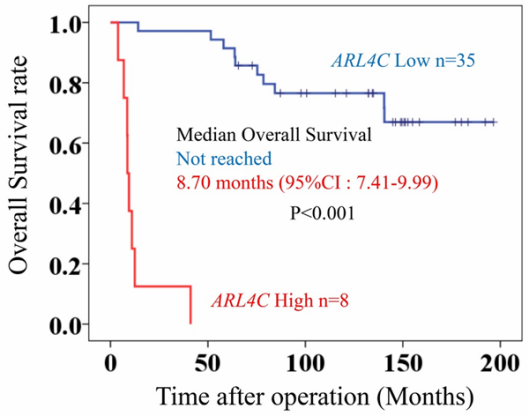

Figure 1. Gene expression levels of *ARL4C* linked to the prognosis of survivals in patients with renal cell carcinoma (RCC): Kaplan-Meier survival curves for *ARL4C* in the first cohort (43 patients with RCC) (**Table 2**). The group showing high expression of *ARL4C* was significantly associated with shorter survival compared with the group showing low expression of *ARL4C* (log-rank test, P<0.001). Patients with RCC were categorized into high or low *ARL4C* expression groups, based on the cut-off FPKM value obtained from their primary tissues.

cance were two-sided and a P<0.05 was defined as statistically significant. OS curves were estimated through the Kaplan-Meier method using log-rank and chi-squared tests were used to assess significance. The univariate and multivariate Cox proportional hazards regression models were used to evaluate independent prognostic effects of the variables with a 95% confidence interval (CI). The proportional hazard assumption in cox model is verified. In OS intervals, disease-specific death events were used as the indicators of the hazard ratio (HR).

Cell lines and cell culture conditions

The RCC cell lines KMRC-1 and SW839 from the American Type Culture Collection were maintained in RPMI 1640 (Nakarai Tesque, Kyoto, Japan), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

siRNA

RNA duplexes for siRNA targeting human ARL4C (*ARL4C*, s223085) and scrambled control RNA duplexes (Silencer® Select Negative Control #1 siRNA, 4390844) were purchased from Life Technologies (Thermo Fisher scientific, Walsham, MA, USA). Cells were transfected with RNA duplexes using Lipofectamine RNAiMAX reagents (Thermo Fisher scientific) according to the manufacturer's protocol.

Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed using the LightCycler 480 SYBG Master I Mix and LightCycler 480 System II (Roche Diagnostics, Mannheim, Germany). Gene expression was normalized using the *GAPDH* gene and standardized using the values obtained from SW839 cells. The primer sequences are provided in

Table 2. Prognostic evaluation of the clinicopathological variables affecting the cancer-specific survival of patients with renal cell carcinoma in the first cohort (n=43)

	n	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p* value†	HR (95% CI)	*p* value†
Gender (male/female)	27/16	2.49 (0.331-18.7)	0.375		
Age (>68/<68)	21/22	1.39 (0.550-3.56)	0.813		
Pathological type (non-clear/clear)	0/43				
pT classification (3,4/1,2)	14/29	4.80 (1.87-12.4)	0.001		
pN classification (x,0/1,2)	41/2	2.61 (1.34-5.06)	0.005		
Vascular invasion (yes/no)	19/24	5.08 (1.79-14.4)	0.002		
Sarcomaid histology (yes/no)	5/38	80.4 (8.99-719)	<0.001		
Histological grade (3/1,2)	2/41	12.5 (2.39-65.3)	0.003		
Nephrectomy (radical/partial)	32/11	8.70 (1.15-65.7)	0.036		
Metastasis at diagnosis (yes/no)	11/32	11.2 (4.12-30.2)	<0.001	7.25 (1.63-32.2)	0.009
ARLC4 expression (high/low)	8/35	111 (12.5-10000)	<0.001	167 (4.71-1000)	0.005

†Cox proportional hazards regression models; HR: hazard ratio; CI: confidence interval.

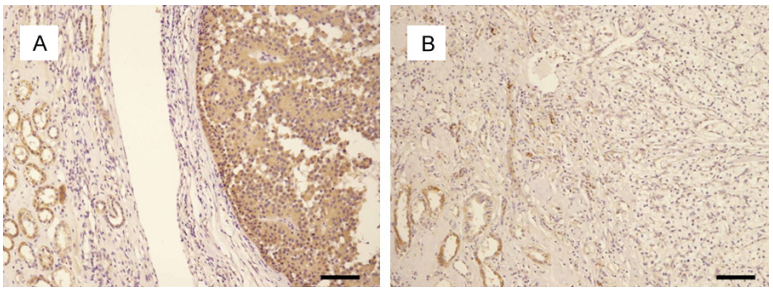

Figure 2. Representative immunohistochemical analysis of ARL4C in samples from primary tissues of patients with renal cell carcinoma (RCC): According to the immunohistochemical intensity of ARL4C, the histological samples were semi-quantitatively categorized into the following two grades; high-expression samples (A) showing equal to or higher intensity of staining versus healthy proximal renal tubules; and low-expression samples (B) showing either negative or lower positive staining versus healthy proximal renal tubules. Normal proximal renal tubules were used as the internal control for each staining specimen. Bars indicate 100 μm.

Table S1. All analyses were performed in triplicate.

In vitro invasion assay

The *in vitro* invasive ability of cancer cells was determined using Matrigel™ Basement Membrane Matrix Invasion Chambers (chamber size: 6.4 mm; membrane surface area: 0.3 cm^2; pore size: 8 μm; BD Biosciences, Bedford, MA, USA) following the manufacturer's instructions. Briefly, 750 μl of culture medium with 10% FCS were added to the plate well as a chemoattractant. Moreover, 500 μl of cell suspension (2 × 10^4 cells/ml) of KMRC-1 cells, previously treated with siRNA for 2 days, without FCS, were added to each chamber. The chambers were incubated for 1 day in a humidified 5% CO_2 atmosphere. Noninvasive cells were removed from the upper surface of the membrane using a cotton swab. The invasive cells on the underside of the membrane were stained with Diff-Quik™ stain (Sysmex Corporation, Kobe, Japan) and counted under a microscope BX-61 (Olympus, Tokyo, Japan). Student's *t*-test was used for the statistical analysis of the results of this assay. Variables with a $P<0.05$ were considered statistically significant.

Results

Global transcriptomic analysis for identification of predictive biomarkers in primary tissues of patients with RCC

The global transcriptional analysis performed in the first cohort showed the following: 27 cases in disease-free status without metastases 5 years after initial surgery for the resection of the primary RCC lesion (Group q1); seven cases with survivals ≥4 years after initial diagnosis of metastasis (Group q2); and nine cases with survivals ≤2 years after initial diagnosis of metastasis (Group q3). A comparative analysis of these three groups was performed, especially focusing on Group q3. In Group q3, we identified 296 and 102 genes which were significantly up-regulated compared with Group q1 and Group q2, respectively. Moreover, we detected

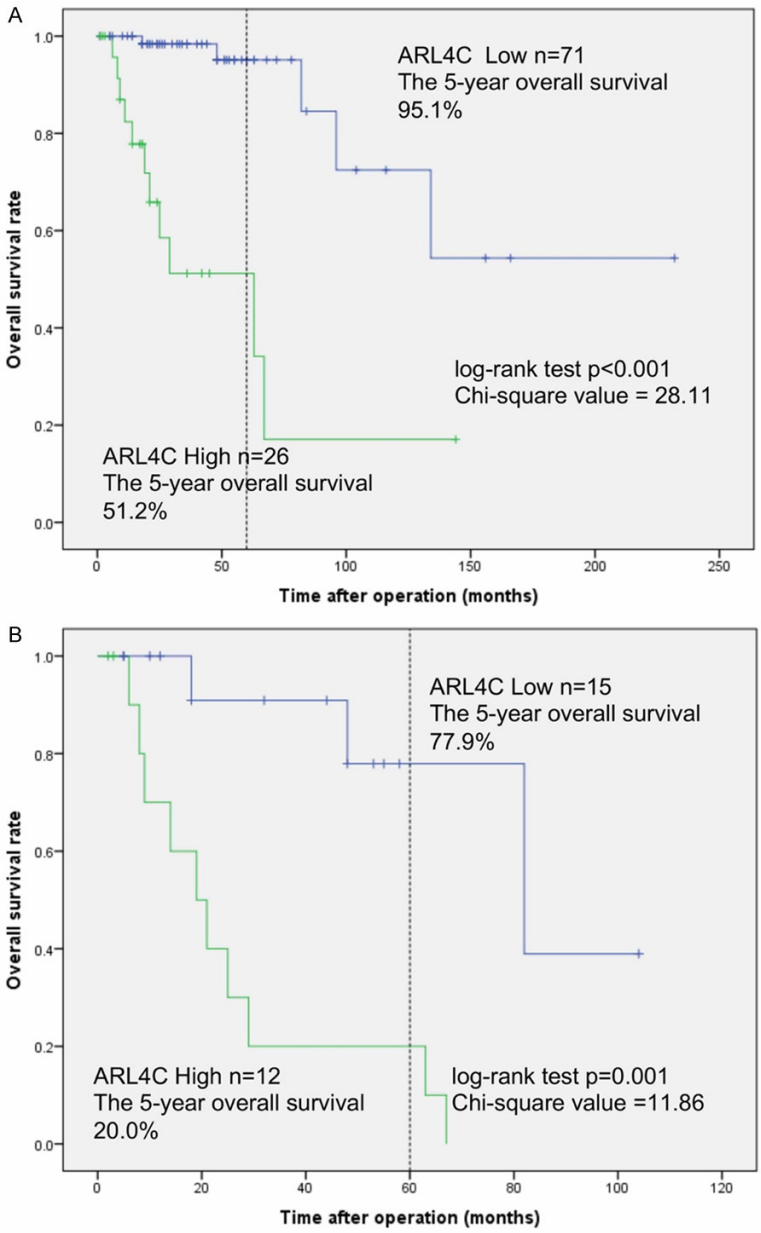

Figure 3. Immunohistochemical levels of ARL4C linked to the prognosis of survival in patients with renal cell carcinoma (RCC): Kaplan-Meier survival curves for ARL4C in the second cohort (97 patients with RCC) (A) and in the subgroup of 27 cases with metastasis at diagnosis (B). (A and B) correspond to **Tables 3** and **4**, respectively. The high-expression group of ARL4C was significantly associated with shorter survival compared with that observed in the low-expression group (log-rank test, A, P<0.001; and B, P=0.001, respectively).

36 genes including 29 cording genes, which were specifically up-regulated only in Group q3 (Table S2). In the first cohort, the ROC curve analysis for each FPKM transcriptional value of 29 genes showed that seven genes were able to predict patient mortality caused by RCC within 2 years after initial diagnosis of metastasis, with an accuracy ≥90% (**Table 1** and Figure S1). These seven genes are potential predictive biomarkers of poor prognosis in patients with RCC.

Predictive value of ARL4C for poor prognosis in patients with RCC

Among the seven genes identified, *ARL4C* showed the best predictive accuracy (**Table 1**). Therefore, the present study evaluated the predictive value of *ARL4C* for poor prognosis in the first cohort. Following the ROC curve analysis, the patients in this cohort were classified into high and low *ARL4C* expression groups, based on the cut-off FPKM value of the primary RCC. The cases with high levels of *ARL4C* expression were linked to significantly shorter survival periods than those observed in the cases with low levels of *ARL4C* expression (log-rank test, P<0.001; 8.7 months vs. not reached, respectively) (**Figure 1**). The Cox univariate and multivariate analyses showed that high levels of *ARL4C* expression accurately predicted poor survivals in this cohort (hazard ratio =111 and 167, P<0.001 and P=0.005, respectively) (**Table 2**). These results showed that *ARL4C* might be a usefully predictive biomarker of poor prognosis in patients with RCC.

Confirmative evaluation of ARL4C as a predictive marker of poor prognosis in patients with RCC

The expression of *ARL4C* was immunohistochemically analyzed in the second independent cohort to confirm its prognostic value. In this

Table 3. Prognostic evaluation of the clinicopathological variables affecting the overall survival of patients with renal cell carcinoma in the second cohort (n=97)

	n	Univariate		Multivariate	
		HR (95% CI)	*p* value†	HR (95% CI)	*p* value†
Sex (male/female)	77/20	0.85 (0.24-3.02)	0.807		
Age, years (>62/≤62)	52/45	0.75 (0.27-2.04)	0.569		
Pathological type (non-clear/clear)	21/76	1.30 (0.36-4.75)	0.690		
T classification (3,4/1,2)	25/72	8.76 (2.90-26.42)	<0.001	3.10 (0.68-14.15)	0.145
Vascular invasion (yes/no)	67/30	36.65 (0.41-3276)	0.116		
Histological grade (3/1,2)	18/79	12.83 (3.93-41.91)	<0.001	1.91 (0.43-8.50)	0.394
Nephrectomy (radical/partial)	51/46	10.27(1.32-80.06)	0.026	1.50 (0.14-16.26)	0.738
Metastasis at diagnosis (yes/no)	27/70	23.31 (5.23-103.8)	<0.01	10.40 (1.76-61.31)	0.010
Molecular targeting therapy (yes/no)	25/72	5.19 (1.88-14.32)	0.001	0.22 (0.09-1.71)	0.399
ARLC4 expression (high/low)	26/71	3.98 (1.43-11.07)	<0.001	9.41 (2.03-43.63)	0.004

†Cox proportional hazards regression models; HR: hazard ratio; CI: confidence interval.

Table 4. Prognostic evaluation of the clinicopathological variables affecting the overall survival of patients with metastatic renal cell carcinoma in the second cohort (n=27)

	n	Univariate		Multivariate	
		HR (95% CI)	*p* value†	HR (95% CI)	*p* value†
Sex (male/female)	21/6	0.68 (0.18-2.56)	0.569		
Age, years (>62/≤62)	15/12	0.50 (0.16-1.60)	0.243		
Pathological type (non-clear/clear)	3/24	3.73 (0.93-15.03)	0.064		
T classification (3,4/1,2)	17/10	1.47 (0.48-4.54)	0.502		
Vascular invasion (yes/no)	26/1	21.74 (0.00-1.2E10)	0.735		
Histological grade (3/1,2)	11/16	3.85 (1.15-12.91)	0.029	1.47 (0.38-5.68)	0.575
Nephrectomy (radical/partial)	26/1	0.21 (0.024-1.89)	0.164		
Molecular targeting therapy (yes/no)	18/9	1.99 (0.54-7.32)	0.300		
ARL4C expression (high/low)	8/19	9.31 (2.02-43.05)	0.004	7.59 (1.40-41.27)	0.019

†Cox proportional hazards regression models; HR: hazard ratio; CI: confidence interval.

analysis, the presence of ARL4C in 97 primary RCC tissue samples was semi-quantitatively evaluated, offering a non-ambiguous evaluation of the expression of ARL4C in tumors. The levels of *ARL4C* were compared with those observed in healthy proximal renal tubules used as internal control on the same slide (**Figure 2**). Twenty-six cases exhibited high expression levels of ARL4C, confirming its association with significantly shorter survivals (**Figure 3A**) (log-rank test, P<0.001) and **Table 3** (Cox multivariate analysis, hazard ratio =9.41, P=0.004). In 27 patients with metastatic RCC, high expression levels of ARL4C were similarly associated with significantly shorter survivals (**Figure 3B**: log-rank test, P=0.001) (**Table 4**; Cox multivariate analysis, hazard ratio =7.59, P=0.019). These results corroborated those of the transcriptomic analysis, showing that *ARL4C* may be a useful biomarker of poor prognosis in patients with RCC.

Discussion

ARL4C was identified as one of seven genes, which demonstrated high accuracy for the prediction of poor prognosis in patients with RCC. The usefulness of evaluating the transcriptional levels of *ARL4C* was also supported using quantitative reverse-transcription-polymerase chain reaction (qRT-PCR). The transcriptional levels of *ARL4C* were similar between the global transcriptomic and qRT-PCR analyses. Furthermore, in RCC samples obtained from Group q3 (i.e., survival ≤2 years after initial diagnosis of metastasis), the expression of *ARL4C* was significantly higher than those reported in the Groups (q1 and q2) surviving ≥5 years without

or ≥4 years with metastases (Figure S2). In addition, a high level of *ARL4C* expression predicted poor survival in the first cohort. Collectively, these results indicated that *ARL4C* may be a useful predictive biomarker of poor prognosis. In addition, the immunohistochemical analysis of ARL4C expression in the second cohort confirmed its predictive value for prognosis. Moreover, these data are consistent with those stored in The Cancer Genome Atlas (TCGA; https://www.proteinatlas.org) database, indicating that *ARL4C* may be a marker of unfavorable prognosis.

In the first cohort, patients with RCC and high expression of *ARL4C* were associated with poor prognosis (i.e., survival ≤2 years), despite treatment with agents targeting tyrosine kinase and mTOR. Similarly, among patients in the second cohort, those with advanced RCC who were treated using molecular targeted therapies and had high expression levels of ARL4C (25 cases) were significantly associated with shorter survival compared with those showing low expression levels (Figure S3; log-rank test, P=0.014) (Table S3; Cox univariate analysis, hazard ratio =5.70, P=0.028). Therefore, RCC patients with high expression levels of ARL4C are resistant to treatment with tyrosine kinase and mTOR inhibitors. Hence, novel therapeutic agents targeting other molecules are required for the treatment of such patients.

Using an *in vitro* Matrigel assay, we showed that siRNA knockdown of *ARL4C* reduced significantly the invasive ability of malignant RCC cells (Figure S4). These findings were consistent with those of a previous report investigating the role of *ARL4C* in colorectal and lung cancer cells [20]. Therefore, ARL4C may be a novel therapeutic target against RCC. However, caution should be exercised in targeting ARL4C in a human clinical setting, considering its expression in normal proximal renal tubules. The use of direct inhibitors of ARL4C may not be applicable *in vivo*. However, the molecules and pathways involved in the expression of ARL4C may represent promising novel therapeutic targets against RCC. Further studies are warranted to confirm the role of ARL4C in RCC.

ARL4C may be a biomarker for the prediction of poor prognosis in patients with RCC and may be a novel target in the development of therapeutics against RCC.

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Disclosure of conflict of interest

None.

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Table S1. Oligonucleotides used for quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

Gene symbol	Forward (5' to 3')	Reverse (5' to 3')
ARL4C	CGTGCGCCATCATCGGCGAG	GCCGGTAAATCAGACTTCGCAG
GAPDH	GGGAGCCAAAAGGGTCATCATC	TGGCATGGACTGTGGTCATGAG

Table S2. The list of 29 genes specifically up-regulated in group q3, through the comparison of group q3 vs. group q1 and group q3 vs. group q2

Gene	q3 vs. q1		q3 vs. q2	
	log2 (fold_change)	q_value	log2 (fold_change)	q_value
ADAMTSL4	2.76966	0.0117264	4.38281	0.00507382
AKR1C2	2.98794	0.00507382	3.67703	0.0224086
ARL4C	2.07493	0.00507382	1.61243	0.0117264
ARNTL2	2.21593	0.00507382	1.56759	0.0416763
ASH1L	1.64754	0.00507382	2.90115	0.00507382
BCAT1	1.6301	0.00507382	1.68479	0.0117264
BIRC5	1.6045	0.00507382	1.52502	0.0224086
BUB1	1.96569	0.00507382	1.25824	0.0265697
C15orf48	3.41135	0.00507382	3.71432	0.00507382
CACNA2D4	1.17443	0.0265697	1.29368	0.0265697
CHI3L2	2.69913	0.00507382	1.89729	0.0200165
COL1A1	1.85073	0.00913033	1.59964	0.0327706
CPD	1.33749	0.0117264	1.55399	0.0265697
CPS1	2.27409	0.00507382	2.74522	0.0200165
DCBLD2	2.70193	0.00507382	2.42609	0.00507382
DGCR8	2.21988	0.00507382	3.89649	0.00507382
DGKI	2.25659	0.00507382	2.12159	0.0117264
ECT2	1.23716	0.00507382	1.19991	0.0117264
EMR2	1.11543	0.00507382	1.22877	0.014572
FN1	2.63834	0.00507382	1.28367	0.0346192
FSTL1	1.76303	0.00507382	1.9964	0.0174473
G3BP2	1.17278	0.0117264	1.70332	0.00507382
HEATR6	1.98954	0.00507382	3.55534	0.00507382
HGF	1.77263	0.00507382	1.96179	0.00913033
IGF2BP1	5.44704	0.00507382	5.2	0.0327706
KCTD12	2.0158	0.00507382	2.19619	0.00507382
STEAP3	2.69778	0.00507382	2.30408	0.00913033
STK33	1.03212	0.00507382	1.23625	0.00507382
TG	2.38457	0.00507382	1.4245	0.014572

Group q1: 27 cases in disease-free status without metastases 5 years after initial surgery for the resection of the primary RCC lesion. Group q2: seven cases with survivals ≥4 years after initial diagnosis of metastasis. Group q3: nine cases with survivals ≤2 years after initial diagnosis of metastasis.

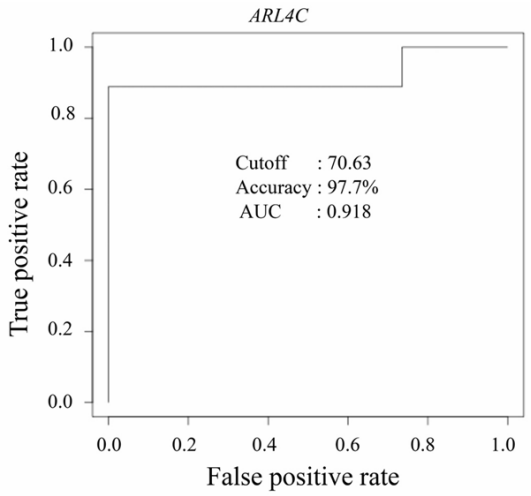

Figure S1. Receiver Operating Characteristic (ROC) curve analysis of the FPKM transcriptional value of *ARL4C* in 43 primary tissues obtained from patients with renal cell carcinoma (RCC): The ROC analysis revealed nine cases with poor prognosis (i.e., survival ≤2 years after initial diagnosis of metastasis) versus 34 cases with good prognosis (i.e., survival ≥5 years without metastases or survival ≥4 years after initial diagnosis of metastasis). *ARL4C* was able to differentiate between cases with poor and good prognosis with an accuracy of 97.7%.

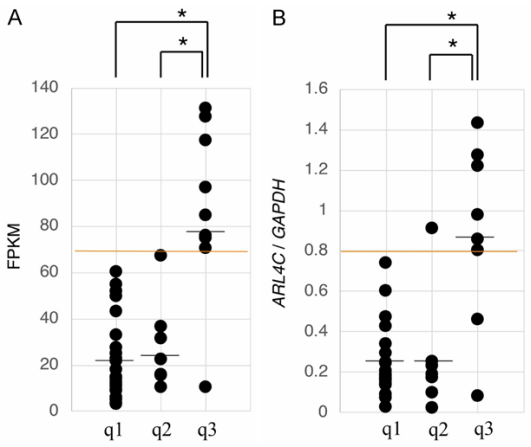

Figure S2. The transcriptional comparison of *ARL4C* between FPKM values through global transcriptomic and quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) analyses in primary tissues obtained from patients with renal cell carcinoma (RCC): FPKM (A) values of *ARL4C* were determined in 43 primary tissues with RCC and the cases were categorized into three Groups: Group q1, 27 cases in disease-free status without any metastasis 5 years after initial surgery for the resection of a primary RCC lesion; Group q2, seven cases with survival ≥4 years after initial diagnosis of metastasis; and Group q3, nine cases with survival ≤2 years after initial diagnosis of metastasis, as shown in **Table 2**. Quantitative RT-PCR (qRT-PCR) (B) values of *ARL4C* were determined in 37 primary tissues with RCC and the cases were categorized into three Groups: Group q1, 22 cases; Group q2, seven cases; and Group q3, eight cases from the 43 cases used in FPKM value analysis. *GAPDH* was used as internal control and the values were standardized using the malignant RCC cell line SW839 [10]. ANOVA: (A) $F(2, 40) = 29.445$, $P = 1.374e^{-8}$; $P<0.05$, (B) $F(2, 34) = 16.104$, $P = 1.201e^{-5}$; $P<0.05$, pair-wise comparisons using t tests with pooled SD vs. q3 (*). Notably, the transcriptional values of *ARL4C* between FPKM and qRT-PCR showed similar patterns. In addition, cases in Group q3 expressed significantly higher levels of *ARL4C* than cases in Groups q1 and q2. Yellow and black lines indicate cut-off value and average, respectively.

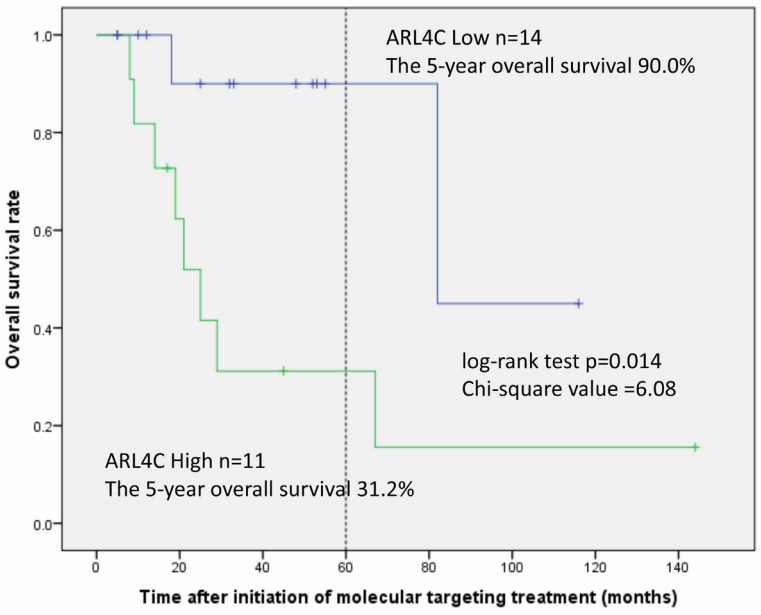

Figure S3. Immunohistochemical levels of ARL4C linked to the prognosis of survival in patients with advanced renal cell carcinoma (RCC): Kaplan-Meier survival curves for ARL4C in 25 cases with advanced RCC followed by molecular targeted therapies among patients in the second cohort. This Figure corresponds to Table S3. The high-expression group of ARL4C was significantly associated with shorter survival compared with those observed in the low-expression groups (log-rank test, P=0.014).

Table S3. Prognostic evaluation of clinicopathological parameters affecting overall survival in patients with advanced RCC followed by molecular targeted therapies in the second cohort (n=25)

	n	Univariate		Multivariate	
		HR (95% CI)	*p* value†	HR (95% CI)	*p* value†
Sex (male/female)	21/4	0.26 (0.62-1.07)	0.061		
Age, years (>62/≤62)	12/13	0.49 (0.14-1.76)	0.273		
Pathological type (non-clear/clear)	5/20	0.36 (0.47-8.25)	0.355		
T classification (3,4/1,2)	16/9	1.45 (0.40-5.21)	0.570		
Vascular invasion (yes/no)	24/1	28.34 (0.003-246638.96)	0.470		
Histological grade (3/1,2)	8/17	3.88 (0.97-15.57)	0.056		
Nephrectomy (radical/partial)	22/3	1.35 (0.16-11.05)	0.782		
ARL4C expression (high/low)	11/14	5.70 (1.20-27.03)	0.028		

†Cox proportional hazards regression models; HR: hazard ratio; CI: confidence interval.

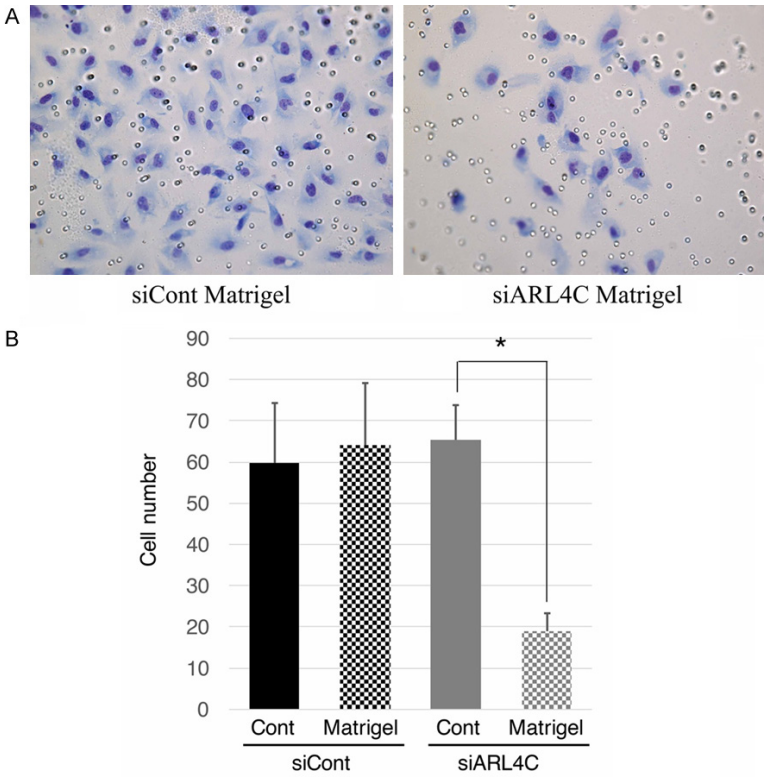

Figure S4. siRNA knockdown of *ARL4C* reduced invasiveness in KMRC-1 renal cell carcinoma: (A) Photographs of invading cells into the Matrigel under treatment with siRNA for *ARL4C* (right) or control (left) in KMRC-1 renal cell carcinoma. Magnification × 100. (B) Scores of invading cell numbers per visual field of a microscope at × 100 magnification. Error bars represent standard errors from independent triplicate experiments. Student's *t*-test (two-tailed) was used to compare with control (siCont). Asterisks indicate P<0.05. Notably, siRNA knockdown of *ARL4C* significantly reduced the invasive ability of malignant renal cell carcinoma in the *in vitro* Matrigel assay.