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ORIGINAL ARTICLE

Mitochondrial PIWI-interacting RNAs are novel biomarkers for clear cell renal cell carcinoma

Chenming Zhao[1,3] · Yuri Tolkach[2] · Doris Schmidt[1] · Marieta Toma[2] · Michael H. Muders[2] · Glen Kristiansen[2] · Stefan C. Müller[1] · Jörg Ellinger[1,4]

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Abstract

Purpose PIWI-interacting RNAs (piRNAs) have been suggested to serve as biomarkers in cancer. In this study, we validated the expression profile of two piRNAs derived from mitochondria, piR-34536 and piR-51810, in tissue and serum of a cohort of clear cell renal cell carcinoma (ccRCC) patients.

Methods Tissue and serum samples of patients with ccRCC were collected prospectively in our biobank. Total RNA was isolated from 118 ccRCC tissues, 75 normal renal tissues as well as 30 serum samples from patients with ccRCC, and 15 serum samples from patients with non-malignant diseases. The expression of piRNAs was determined using quantitative real-time PCR.

Results Both piR-34536 and piR-51810 were downregulated in ccRCC compared to non-malignant renal tissue. Decreased tissue piRNA levels were significant and independent predictors of shortened progression-free, cancer-specific and overall survival of ccRCC patients. The piRNA levels in serum did not differ in ccRCC patients and control subjects.

Conclusions The expression of piR-34536 and piR-51810 in ccRCC tissues may be used as prognostic biomarkers in ccRCC.

Keywords Clear cell renal cell carcinoma · piRNA · Biomarker · Mitochondrial

Introduction

Renal cell carcinoma (RCC) is a common malignancy, representing 2–3% of all cancers, with an increasing incidence over the last decades [1]. Clear cell renal cell carcinoma (ccRCC) is the most frequent histological RCC subtype, with a worse prognosis compared to papillary or chromophobe RCC [2]. Despite diagnostic advances in recent years, especially improved imaging techniques, a significant proportion of renal masses removed for suspected malignancy are histologically benign [3]. Unfortunately, after curative treatment, such as radical or partial nephrectomy, up to 30% of patients with localized RCC develop recurrence within 5 years [4]. The prognosis is poor for patients with metastatic RCC; the 5-year survival rate after diagnosis is only 8% [5]. Predictive factors are essential to guide individualized management; however, biomarkers are not yet available for daily routine [6]. Novel biomarkers for ccRCC patients are, therefore, urgently warranted.

Small non-coding RNAs (sncRNA) such as microRNA, tRNA, rRNA, snoRNA and YRNA have attracted interest of cancer biomarker researchers within the last decade. Several sncRNAs have specific functions in oncogenesis and cancer progression [7]. In recent years, a novel class of sncRNA, P-element induced wimpy testis (PIWI)-interacting RNA (piRNA) has been recognized. The piRNAs interact with the PIWI protein family and are implicated in gene regulation and silencing [8]. Moreover, a number of piRNAs are located in mitochondria and differently distributed in various cancer cells [9]. According to a database of small RNA sequencing in human cancer diseases (http://ngs.ym.edu.tw/ym500/), some piRNAs are reported dysregulated in ccRCC

✉ Jörg Ellinger
joerg.ellinger@ukbonn.de

1 Department of Urology, University Hospital Bonn, Bonn, Germany

2 Department of Pathology, University Hospital Bonn, Bonn, Germany

3 Department of Urology, Second Hospital of Hebei Medical University, Shijiazhuang, China

4 Klinik und Poliklinik für Urologie und Kinderurologie, Universitätsklinikum Bonn, Sigmund-Freud-Strasse 25, 53127 Bonn, Germany

tissues [10]. Among them, piR-34536 and piR-51810 are derived from mitochondrial DNA. In this study, we investigated the expression profile of these two piRNAs in a ccRCC cohort to evaluate the diagnostic and prognostic potential utility of mitochondrial piRNAs.

Materials and methods

Sample collection

Serum and tissue samples were collected prospectively in our institutional biobank at the CIO Cologne–Bonn from patients with renal tumors treated with partial or radical nephrectomy. Tissues were fresh frozen and stored at − 80 °C until use. All samples were reviewed by an experienced uro-pathologist to ensure a fraction of at least 80% tumor cells in the investigated specimens and to confirm stage and grade. Serum samples were collected from patients with ccRCC; samples from patients with non-malignant urological diseases (benign prostate hyperplasia or urinary incontinence) were used as control. Blood was drawn preoperatively in Serum S-Monovette gel tubes with clotting activator. Serum was collected and stored in cryotubes at − 80 °C.

In total, 193 RNA samples (118 ccRCC, 75 normal) from tissues and 45 RNA samples (30 ccRCC, 15 normal) from serum were studied. The samples were selected randomly from the biobank. Follow-up information of revisiting patients was obtained from the hospital information system. Survival data were available for a subset of 105 ccRCC patients with a mean follow-up time of 35.7 (range 1–146) months. The clinicopathological information of the patients recruited for this study is provided in Table 1. The study was approved by the ethic committee of University Bonn.

RNA isolation

Total RNA was isolated from 50 mg renal tissue using the mirVana miRNA Isolation Kit (Ambion) and treated twice with deoxyribonuclease (DNA-free Kit, Ambion) as described before [11]. Serum RNA was isolated as published earlier using the mirVana Paris-Kit (Ambion) from 400 µl serum [12]. All procedures were performed according to the manufacturers' recommendations. RNA quantity was measured using a NanoDrop 2000 spectrophotometer

Table 1 Summary of clinicopathological parameters of the study cohorts

	Tissue cohort		Serum cohort	
	ccRCC $N=118$ (%)	Control $N=75$ (%)	ccRCC $N=30$ (%)	Control $N=15$ (%)
Gender				
Male	81 (68.6)	52 (69.3)	18 (60.0)	7 (46.7)
Female	37 (31.4)	23 (30.7)	12 (40.0)	8 (53.3)
Age				
Mean (range)	65.5 (36–89)	63.4 (35–89)	63.2 (42–82)	59.9 (41–79)
TNM stage		Not applicable		Not applicable
T1	68 (57.6)		15 (50.0)	
T2	10 (8.5)		0 (0)	
T3	37 (31.4)		15 (50.0)	
T4	3 (2.5)		0 (0)	
Distant metastasis/M1	17 (14.4)		1 (3.3)	
Stage grouping		Not applicable		Not applicable
Stage I	62 (52.5)		15 (50.0)	
Stage II	7 (5.9)		0 (0)	
Stage III	30 (25.4)		15 (50.0)	
Stage IV	19 (16.1)		0 (0)	
ISUP grade		Not applicable		Not applicable
Grade 1	12 (10.2)		1 (3.3)	
Grade 2	81 (68.6)		25 (83.3)	
Grade 3	19 (16.1)		4 (13.3)	
Grade 4	6 (5.1)		0 (0)	
Recurrence	29 (24.6)	Not applicable	0 (0)	Not applicable
Death from cancer	16 (13.6)	Not applicable	0 (0)	Not applicable

(Thermo Scientific) and RNA integrity was confirmed by gel electrophoresis.

Quantitative real-time PCR

cDNA was synthesized with 500 ng RNA using the miScript II RT Kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) was performed with 1 ng/µl cDNA (tissue) or 1 µl (serum) cDNA template using Qiagen miScript SYBR Green PCR technology (Hilden, Germany). Pre-designed Qiagen miScript Primer Assays were used to quantify the reference gene SNORD43 (MS00007476) and RNU6-2 (MS00033740); custom-made miScript Primer Assays were used for the target genes piR-34536 (YCP0032199) and piR-51810 (YCP0032205). The QuantStudio 5 real-time PCR system was used to incubate the PCR mixture at 95 °C for 15 min, and then 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. Relative piRNA levels were calculated using RNU6-2 and SNORD43 for both tissue and serum samples as endogenous reference genes, with QuantStudio 3D Analysis Suite Cloud software.

Statistical analysis

All statistical analyses were performed with SPSS Statistics v22 (IBM, Ehningen, Germany). The Mann–Whitney *U* test was used to compare the piRNA expression with clinicopathological parameters. The area under curve (AUC) of the receiver operating characteristic (ROC) curves was employed to estimate the discriminative sensitivity and specificity of piRNA expression. To determine the optimal cutoff, the Youden index based on ROC curves was calculated for each variable. For survival analysis, Kaplan–Meier curves, log-rank test and Cox proportional regression analysis were applied. Statistical significance was considered at $p < 0.05$.

Results

Diagnostic relevance of piRNA expression

The expression levels of both piR-34536 and piR-51810 were significantly decreased in ccRCC compared to normal tissue (both $p < 0.001$). The ROC analyses indicated that they had a diagnostic potential with an AUC of 0.815 (95% CI 0.751–0.875) for piR-34536, and AUC of 0.829 (95% CI 0.765–0.892) for piR-51810. The Youden index was applied to select the optimum cutoff point: piR-34536 had a sensitivity of 78.0% and a specificity of 78.1%, piR-51810 achieved a sensitivity of 85.6% and a specificity of 71.2%; see Fig. 1.

The circulating piRNA levels were measured in serum. Both piR-34536 and piR-51810 were detectable in serum. However, we did not observe significant differences between ccRCC patients and the control subjects (Fig. 2).

Prognostic relevance of piRNA expression

We also analyzed the association of piRNA expression in tissue with clinicopathological characteristics. piR-51810 was significantly decreased ($p = 0.003$) in primary tumors of patients with metastatic ccRCC compared to patients with non-metastatic ccRCC (cM0); see Fig. 3. Given the small number of patients staged pT2 ($n = 10$) and pT4 ($n = 3$), we decided to combine pT1 and pT2, as well as

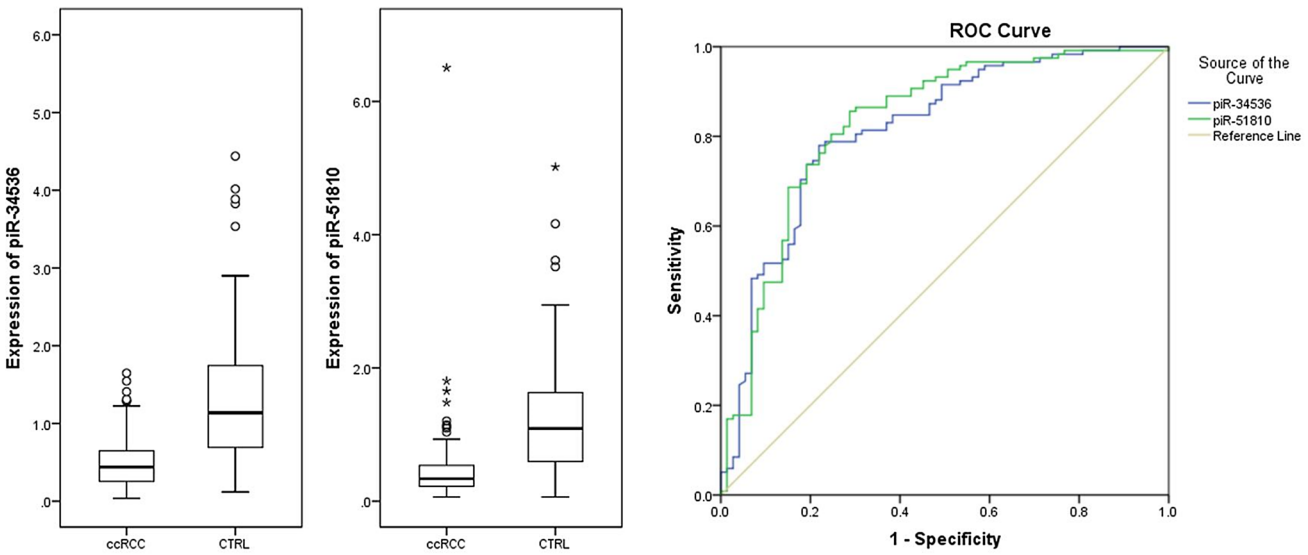

Fig. 1 The expression of piR-34536 ($p < 0.001$) and piR-51810 ($p < 0.001$) is decreased in ccRCC compared to normal renal (CTRL) tissues

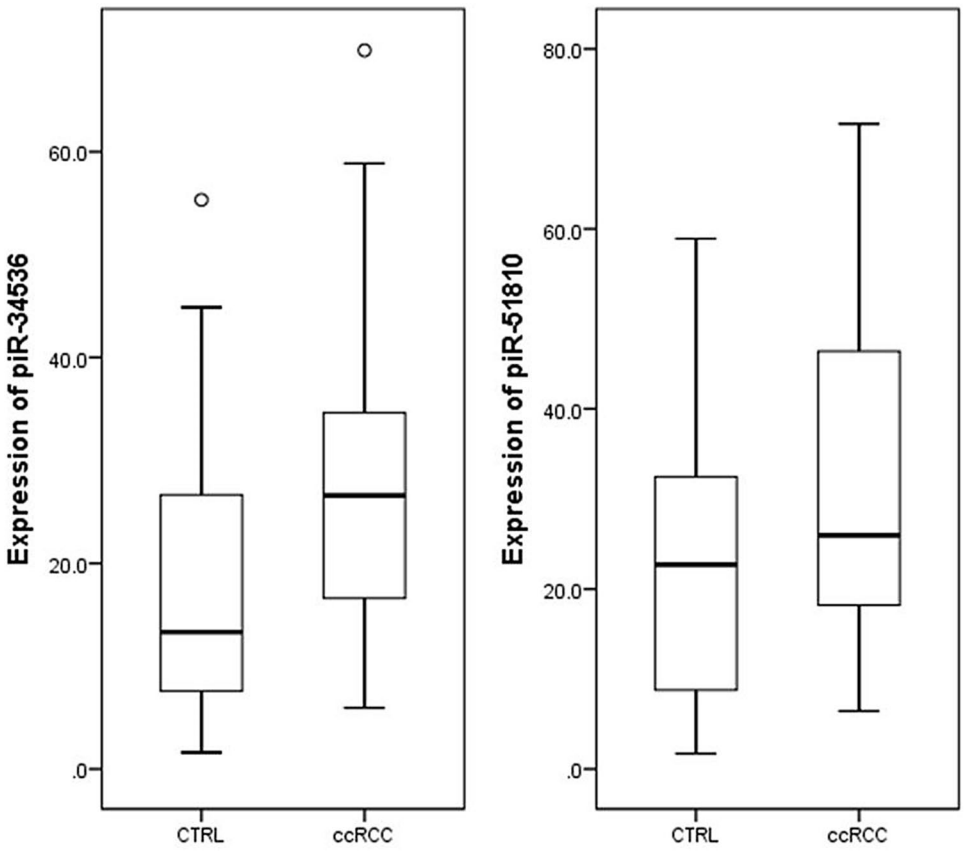

Fig. 2 The levels of circulating piRNAs are similar in serum of patients with ccRCC compared to patients with non-malignant urological diseases (CTRL). piR-34536 $p = 0.092$; piR-51810 $p = 0.268$

pT3 and pT4 patients for the correlation of pT-stage with piRNA expression levels. Neither stage nor grade was correlated with piRNA expression levels (all $p > 0.05$).

To determine the relevance of piRNA expression as a prognostic parameter, we correlated piRNA expression levels with progression-free survival (local relapse or novel metastasis as endpoints), cancer-specific survival and overall survival. In general, lower levels of piR-34536 or piR-51810 were associated with a significant (all $p < 0.05$) shorter progression-free, cancer-specific as well as overall survival period (see Fig. 4). Univariate Cox regression analyses indicated that both piR-34536 and piR-51810 were significant prognostic factors for progression-free, cancer-specific and overall survival. For the multivariate cox regression analysis, we used model 1 for piR-34536 and model 2 for piR-51810; both models were adjusted to age, pT-stage, M-stage and pathological grade, respectively. Except piR-34536, which failed to predict independently in progression-free survival, the studied piRNAs retained their prognostic value in the multivariate models for prediction of patients' survival. See Tables 2, 3 and 4.

Discussion

PIWI proteins themselves have been extensively studied in cancer before [13] and recent studies focused on the biological function of piRNA or piRNA/PIWI complexes in human cancers. It is expected that the dysregulation of piRNAs may contribute to the inhibition of tumor suppressor genes or oncogenes by targeting mRNA transcripts [14]. Additionally, piRNAs are able to induce aberrant DNA methylation at special gene sites, thereby leading to changed phenotypes of cancer cells [15]. Moreover, piRNAs can also activate gene expression by upregulation of H3K4me3 and inhibition of H3K27me3 [16]. Finally, piRNAs have also been linked to several cellular functions including cell cycle regulation and DNA synthesis [8], which are crucial for proliferation. Mitochondrial dysfunction plays a key role in tumorigenesis of RCC and the expression of PIWI proteins or piRNAs derived from mitochondria might also alter during cancer progression [9, 17]. Aberrant piRNA expression has been identified in various tumors including breast cancer, gastric cancer,

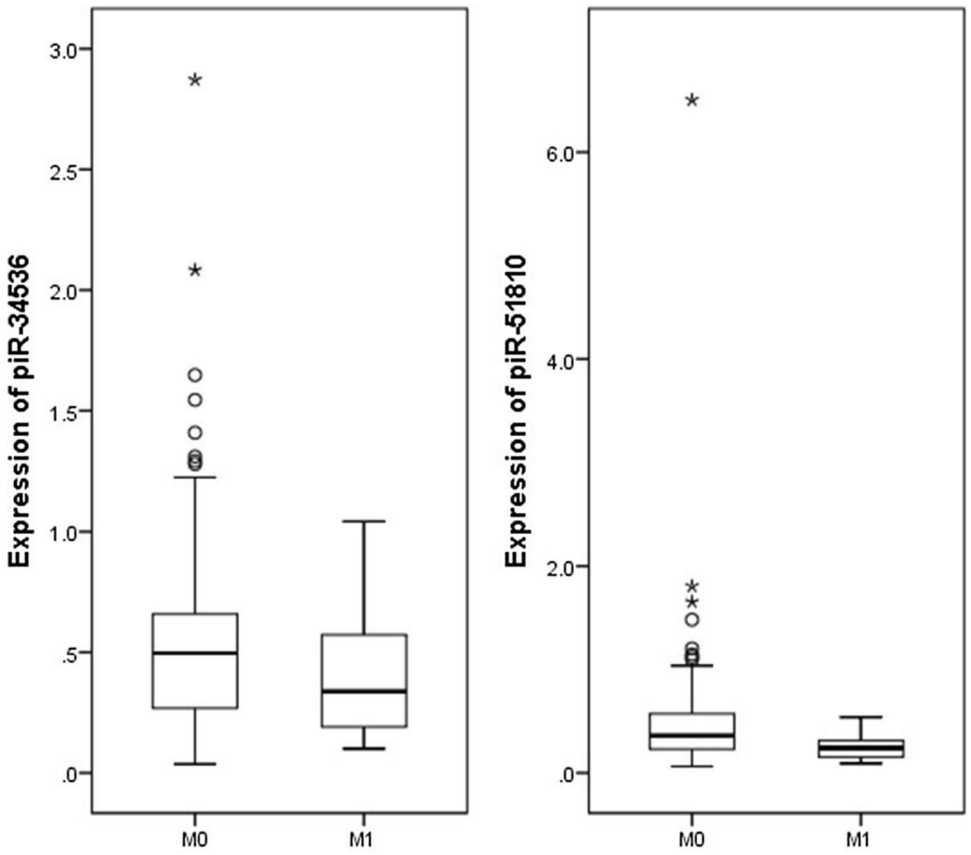

Fig. 3 The expression level of piRNAs in tissues was lower in M1 vs. M0 disease (piR-34536 $p=0.139$; piR-51810 $p=0.003$)

liver cancer, pancreatic cancer, multiple myeloma and lung cancer in a tissue-specific pattern [18]. Previous studies on piRNAs in ccRCC reported upregulation of piR-32051, piR-39894 and piR-43607, whereas other piRNAs like piR-823, piR-38756, piR-57125, and piR-30924 were downregulated in tumor tissues [19–21]. In this study, our interest focused on piR-34536 and piR-51810, which are both derived from mitochondria. Mitochondrial piRNAs are most likely derived from mitochondrial tRNAs and probably associated with stress responses [9]. In agreement with the finding of downregulated piR-34536 and piR-51810 in ccRCC, our earlier study also demonstrated lower levels of stress-induced tRNA-halves in ccRCC [12]. Interestingly, a previous study identified increased expression of three piRNAs from the same piRNA cluster on chromosome 17 in ccRCC [19], thereby indicating that piRNAs within the same cluster sequence may have a similar expression pattern, whereas piRNAs from different clusters may differ in expression in one specific tissue.

In our study, patients with synchronous metastases had significantly lower levels of piR-51810. Unfortunately, our cohort did not allow drawing meaningful conclusions on piR-51810 in patients who developed metachronous metastases. However, we assume that piR-51810 is an independent prognostic biomarker because piRN-51810 expression levels still predicted the outcomes like progression-free, cancer-specific and overall survival in the multivariate models which included pT-stage, M-stage and pathological grade. The relevance of piR-34536 as prognostic marker is less clear: although piR-34536 was a significant predictor for progression-free, cancer-specific and overall survival in the univariate model, it lost its predictive value in the multivariate model for the prediction of progression-free survival. Nevertheless, piR-34536 was still predictive for cancer-specific and overall survival in the multivariate Cox regression analyses. Taken together, mitochondrial piRNAs are attractive biomarker candidates for the prediction of patients' outcome following nephrectomy. Furthermore, other researchers also reported a prognostic relevance for several other piRNAs (e.g., piR-30924 and piR-57125 [20]; pi-823 [21]; piR-32051, piR-39894, piR-43607 [19]); thus, future systematic analysis of the piRNAs class may reveal other interesting molecular biomarkers for the prediction of ccRCC patients' outcome.

Besides, piRNAs are very stable molecules in circulation and have the ability to resist quick degradation in various conditions [22]. One study reported increased piR-823 levels in serum and urine samples from RCC patients, which was in contrast to differences they found in tissue samples [21]. In our study, no significant difference was found in

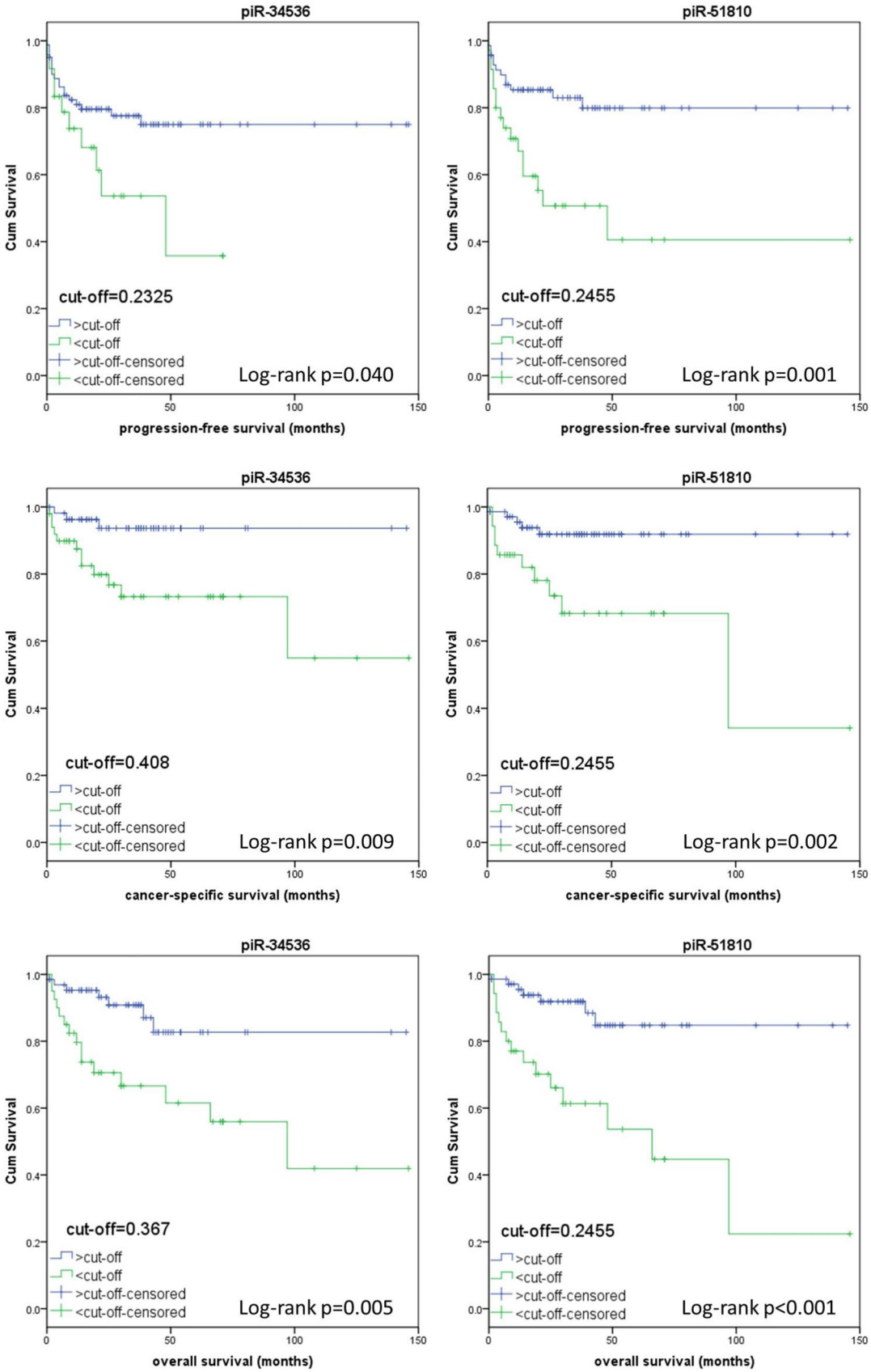

Fig. 4 Kaplan–Meier estimates of piRNAs expression in ccRCC tissue for progression-free survival, cancer-specific survival and overall survival

Table 2 Cox regression analyses of progression-free survival

	Univariate		Multivariate model 1		Multivariate model 2	
	p	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)
Age	0.595	1.009 (0.976–1.043)	0.539	1.011 (0.977–1.046)	0.503	1.012 (0.978–1.047)
T1/2 vs. T3/4	**< 0.001**	0.227 (0.104–0.496)	**0.027**	0.359 (0.144–0.892)	**0.018**	0.342 (0.141–0.830)
M0 vs. M1	**< 0.001**	0.096 (0.045–0.206)	**< 0.001**	0.136 (0.053–0.353)	**< 0.001**	0.175 (0.066–0.463)
Grades 1/2 vs. 3/4	**0.005**	0.331 (0.153–0.715)	0.925	0.957 (0.377–2.426)	0.639	0.805 (0.325–1.994)
piR-34536 > cutoff vs. < cutoff	**0.047**	0.455 (0.209–0.989)	0.244	0.620 (0.277–1.385)		
piR-51810 > cutoff vs. < cutoff	**0.002**	0.308 (0.145–0.654)			**0.043**	0.431 (0.190–0.975)

Statistically significant results are shown in bold

Table 3 Cox regression analyses of cancer-specific survival

	Univariate		Multivariate model 1		Multivariate model 2	
	p	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)
Age	**0.029**	1.063 (1.006–1.123)	**0.024**	1.080 (1.010–1.156)	**0.022**	1.071 (1.010–1.137)
T1/2 vs. T3/4	0.065	0.383 (0.138–1.063)	0.957	1.035 (0.295–3.631)	0.717	0.808 (0.255–2.557)
M0 vs. M1	**< 0.001**	0.133 (0.048–0.368)	**0.006**	0.124 (0.029–0.542)	**0.011**	0.173 (0.045–0.670)
Grades 1/2 vs. 3/4	**0.022**	0.286 (0.098–0.835)	0.769	0.811 (0.200–3.290)	0.955	0.961 (0.239–3.867)
piR-34536 > cutoff vs. < cutoff	**0.017**	0.215 (0.060–0.764)	**0.011**	0.189 (0.052–0.682)		
piR-51810 > cutoff vs. < cutoff	**0.006**	0.220 (0.075–0.646)			**0.033**	0.287 (0.091–0.906)

Statistically significant results are shown in bold

Table 4 Cox regression analyses of overall survival

	Univariate		Multivariate model 1		Multivariate model 2	
	p	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)
Age	**0.021**	1.054 (1.008–1.102)	**0.008**	1.069 (1.017–1.124)	**0.008**	1.066 (1.017–1.117)
T1/2 vs. T3/4	0.095	0.485 (0.208–1.135)	0.911	1.059 (0.384–2.918)	0.851	0.895 (0.284–2.823)
M0 vs. M1	**< 0.001**	0.209 (0.089–0.492)	**0.008**	0.214 (0.068–0.666)	**0.016**	0.258 (0.086–0.773)
Grades 1/2 vs. 3/4	**0.048**	0.395 (0.157–0.994)	0.574	0.721 (0.231–2.254)	0.851	0.895 (0.284–2.823)
piR-34536 > cutoff vs. < cutoff	**0.008**	0.295 (0.119–0.732)	**0.006**	0.275 (0.109–0.693)		
piR-51810 > cutoff vs. < cutoff	**0.001**	0.205 (0.084–0.504)			**0.002**	0.223 (0.087–0.573)

Statistically significant results are shown in bold

serum samples between patients with ccRCC and patients with non-malignant diseases. However, it should be noted that the number of patients in the serum study was low (45 patients). There is no definitive explanation how piRNAs are released into circulation and whether these mechanisms are different in cancer and normal cells. Furthermore, the background level of serum piRNAs derived from non-malignant cells may impede recognizing small expression differences.

In summary, we demonstrate that mitochondria-derived piR-34536 and piR-51810 are potential prognostic biomarker in ccRCC patients. However, there are still some inherent limitations in our study: We did not investigate all the mitochondrial piRNAs in ccRCC. Furthermore, piR-34536 and piR-51810 were not measured in non-ccRCC subtypes. It should also be noted that we isolated RNA from a single tumor foci (with a diameter of approximately 10 mm); thus,

intra-tumor heterogeneity is not well represented. Given the importance of tumor heterogeneity and its clinical relevance in ccRCC [23, 24], further investigations are still required in cell and animal models to determine the biological function and expression pattern of piR-34536 and piR-51810.

Conclusions

Two novel potential prognostic biomarkers were identified for ccRCC: mitochondrial piR-34536 and piR-51810 levels provide additional information for the prognosis with regard to several clinical endpoints and could thereby optimize the individual treatment and thereby finally improve patients' survival.

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Author contributions CZ project development, data collection, manuscript writing. YT data collection, manuscript editing. DS data collection, data analysis. MT manuscript writing and editing. MM manuscript writing and editing. GK project development, manuscript editing. SM project development, manuscript editing. JE project development, data analysis, manuscript editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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