Ann Surg Oncol (2019) 26:3765–3773
https://doi.org/10.1245/s10434-019-07578-3

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – UROLOGIC ONCOLOGY

4-miRNA Score Predicts the Individual Metastatic Risk of Renal Cell Carcinoma Patients

Joana Heinzelmann, PhD[1,2], Madeleine Arndt[1], Ramona Pleyers, MD[1], Tobias Fehlmann[3], Sebastian Hoelters, PhD[1,9], Philip Zeuschner, MD[1], Alexander Vogt[1], Alexey Pryalukhin, MD[4,10], Elke Schaeffeler, PhD[5,6], Rainer M. Bohle, MD, PhD[4], Mieczyslaw Gajda, MD[7], Martin Janssen, MD[1], Michael Stoeckle, MD, PhD[1], and Kerstin Junker, MD, PhD[1,8]

[1]Department of Urology and Pediatric Urology, Saarland University, Homburg, Saar, Germany; [2]Department of Ophthalmology, Martin-Luther University Halle-Wittenberg, University Hospital Halle (Saale), Halle (Saale), Germany; [3]Department of Clinical Bioinformatics, Saarland University, Saarbruecken, Germany; [4]Institute of Pathology, Saarland University, Homburg, Saar, Germany; [5]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany; [6]University of Tuebingen, Tuebingen, Germany; [7]Institute of Pathology, Jena University Hospital, Jena, Germany; [8]Department of Urology, Jena University Hospital, Jena, Germany; [9]*Present Address:* SERVA Electrophoresis GmbH, Heidelberg, Germany; [10]*Present Address:* Institute of Pathology, Bonn University Medical School, Bonn, Germany

ABSTRACT

Background. In order to improve individual prognostication as well as stratification for adjuvant therapy in patients with clinically localized clear cell renal cell carcinoma (ccRCC), reliable prognostic biomarkers are urgently needed. In this study, microRNAs (miRNAs) have emerged as promising candidates. We investigated whether a combination of differently expressed miRNAs in primary tumors can predict the individual metastatic risk.

Methods. Using two prospectively collected biobanks of academic centers, 108 ccRCCs were selected, including 57 from patients with metastatic disease at diagnosis or during follow-up and 51 without evidence of metastases. Fourteen previously identified candidate miRNAs were tested in 20 representative formalin-fixed and paraffin embedded samples in order to select the best discriminators between metastatic and nonmetastatic ccRCC. These miRNAs were approved in 108 tumor samples. We evaluated the association of altered miRNA expression with the metastatic potential of tumors using quantitative polymerase chain reaction. A prognostic 4-miRNA model has been established using a random forest classifier. Cox regression analyses were performed for correlation of the miRNA model and clinicopathological parameters to metastasis-free and overall survival.

Results. Nine miRNAs indicated significant expression alterations in the small cohort. These miRNAs were validated in the whole cohort. The established 4-miRNA score (miR-30a-3p/-30c-5p/-139-5p/-144-5p) has been identified as a superior predictor for metastasis-free survival (hazard ratio 12.402; $p = 7.0E{-}05$) and overall survival ($p = 1.1E{-}04$) compared with clinicopathological parameters, and likewise in the Leibovich score subgroups.

Conclusions. We identified a 4-miRNA model that was found to be superior to clinicopathological parameters in accurately predicting individual metastatic risk and can support patient selection for risk-stratified follow-up and adjuvant therapy studies.

Clear cell renal cell carcinoma (ccRCC) remains the most common renal cell carcinoma (RCC) subtype, representing 75–80% of all cases.[1] The prognosis of ccRCC strongly depends on the development of distant metastases. Approximately 20% of patients experience metastatic disease at the time of initial diagnosis (synchronous

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-019-07578-3) contains supplementary material, which is available to authorized users.

First Received: 10 January 2019;
Published Online: 3 July 2019

K. Junker, MD, PhD
e-mail: kerstin.junker@uks.eu

metastases), while another 20–30% develop metastases after resection of the primary tumor (metachronous metastases).[2,3] Patients affected by metastatic ccRCC have a very poor prognosis, with $a < 10\%$ 5-year survival rate. To date, current guidelines do not advise adjuvant therapy since clinical trials, even in patients with high-risk disease, failed to show an overall survival benefit (European Association of Urology [EAU] guidelines). Hence, reliable prognostic parameters for prediction of the development of metastases are urgently needed to accurately select patients with aggressive RCCs for the design of adjuvant clinical trials or for risk-adjusted follow-up schedules. The Leibovich score, which is based on clinical and pathological parameters,[4] is commonly used to predict the postoperative risk of metastases in RCC patients; however, clinicopathological features do not accurately predict individual disease outcome.[5] Biomarker profiles are likely to reflect the individual biological aggressiveness and metastatic potential of ccRCC more precisely than established clinical or pathologic parameters.[6–10]

Apart from genetic alterations, microRNAs (miRNAs) have a significant potential as biomarkers for individual diagnosis, prognosis, and prediction of therapy response.[11,12] miRNAs regulate gene expression in a post-transcriptional manner and thereby influence a wide variety of cellular processes, including multiple oncogenic pathways.[13]

In two small series of patients, we previously reported an altered expression of 14 miRNAs in tumors with high metastatic potential underlying the prognostic value of miRNAs.[14,15] The aim of the current study was to validate these results in an independent patient cohort.

MATERIALS AND METHODS

Patient Samples

We retrospectively selected 108 primary tumor samples of ccRCC from partial or radical nephrectomy specimens (2000–2013) from two academic centers—Saarland University Medical Center, Homburg, and the Jena University Hospital, Germany. This study included 51 tumor samples from patients who did not develop metastases within at least 4 years after surgery and 57 samples from patients with metastases diagnosed at the time of primary tumor resection (synchronous metastasis, $n = 22$) or in the course of disease (metachronous metastases, $n = 35$). Formalin-fixed and paraffin embedded (FFPE) tissue samples were provided for miRNA analyses. Paraffin sections of each specimen were reviewed by a pathologist, histologically classified according to the WHO classification (2014), and staged according to the Union for International Cancer Control (UICC) TNM classification (2010). Samples were taken from areas with more than 85% of tumor cells and with minimal hemorrhage and no necrosis. Additionally, 10 FFPE samples from nonmalignant kidney tissues of nephrectomy specimens were included as controls. The study was approved by the local Ethics Committee.

Total RNA Isolation

Seven to 10 slices (4–10 μm) of tissue sample bearing $\geq 85\%$ of tumor cells, as evidenced by hematoxylin and eosin (HE) staining, were prepared using a microtome. Total RNA was isolated from macrodissected FFPE samples using an miRNeasy FFPE isolation kit (Qiagen) according to the manufacturer's instructions. Total RNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer.

MicroRNA Expression Analyses

MiRNA expression analyses were performed in two steps. First, we selected the most promising candidates out of 14 previously described miRNAs, based on the results of 20 ccRCC samples (10 nonmetastatic, 10 metastatic ccRCCs) and 10 nonmalignant kidney specimens. Second, we validated the selected miRNAs in the whole cohort of 108 tumor samples.

We generated complementary DNA (cDNA) by reverse transcription (RT) of 100 ng total RNA using a TaqMan MicroRNA Reverse Transcription Kit and specific TaqMan Primers (ThermoFisher) according to the manufacturer's instructions. RT products were preamplified using the TaqMan PreAmp Master Mix Kit (ThermoFisher) according to the manufacturer's instructions, and analyzed in triplicate using a TaqMan Gene Expression Master Mix (ThermoFisher) on a BioMark system (Fluidigm) in the small cohort and on a Step One Plus system (Applied Biosystems) in the whole cohort according to the manufacturer's instructions. MiRNA expression levels were normalized using RNU48. In previous work, we identified RNU48 as the most stable endogenous reference gene in renal cell cancer tissue.

Statistical Analyses

Quantitative polymerase chain reaction (qPCR) expression analyses were performed using the comparative CT method ($2^{\Delta\Delta}$CT) with efficiency correction for RNU48 and miRNAs. Correlation analyses with clinicopathological parameters were conducted using IBM SPSS Statistics version 23 (IBM Corporation, Armonk, NY, USA). The Mann–Whitney U test was performed to compare the

expression levels of miRNAs in renal tissues depending on the clinicopathological parameters. The best differentiating cut-off values of each miRNA to discriminate non-metastatic and metastatic ccRCCs were determined using receiver operating characteristic (ROC) analysis and the Youden Index.

Based on the normalized expression values of the miRNAs, we established a prediction model using a random forest classifier, and assessed its performance using five-times-repeated stratified tenfold cross-validation. Due to the limited amount of FFPE tissue from 23 samples, 85 samples were used for the classifier. Clinical characteristics are summarized in Table 1.

Kaplan–Meier analysis was used to estimate the differences in metastasis-free survival (time between primary tumor surgery and diagnosis of distant metastases) and overall survival (time between tumor nephrectomy and death from any cause) for each miRNA and the 4-miRNA prognostic model. If no metastases were observed during follow-up, metastasis-free survival was censored at the time of last follow-up or death. Univariate/multivariate Cox proportional hazards regression analyses were performed to calculate the prognostic value of the 4-miRNA model for metastasis-free survival. Two-sided statistical tests were used, and C-indices were determined by calculation of ROC curves. Results were determined to be significantly different if p values were $\leq$ 5.0E−02.

RESULTS

The expression analyses using qPCR revealed a significantly decreased expression in metastatic tumors compared with nonmetastatic tumors for 9 of 14 previously described miRNAs (electronic supplementary Table S1). These nine miRNAs (miR-10b-5p, miR-30a-3p, miR-30e-

TABLE 1 Clinicopathological data of ccRCC samples included in the prognostic 4-miRNA model

	Whole cohort	Nonmetastatic tumors	Synchronous metastatic tumors	Metachronous metastatic tumors
No. of patients	85	42	18	25
Age, years [mean/median (range)]	62/62 (39–82)	59/58 (39–79)	67/68 (49–82)	63/62 (41–80)
Sex [male/female (%)]	60/25 (70.6/29.4)	29/13 (69.0/31.0)	10/8 (55.6/44.4)	21/4 (84.0/16.0)
T category [*n* (%)]				
pT1a/b	48 (56.5)	36 (85.7)	1 (5.6)	11 (44.0)
pT2	6 (7.1)	1 (2.4)	2 (11.1)	3 (12.0)
pT3a/b	28 (32.9)	5 (11.9)	14 (77.8)	9 (36.0)
pT4	3 (3.5)	–	1 (5.6)	2 (8.0)
Grade [*n* (%)]				
G1	8 (9.4)	7 (16.7)	–	1 (4.0)
G2	65 (76.5)	34 (81.0)	13 (72.2)	18 (72.0)
G3	11 (12.9)	1 (2.4)	4 (22.2)	6 (24.0)
G4	1 (1.2)	–	1 (5.6)	–
Nodal status [*n* (%)]				
N0	75 (88.2)	42 (100.0)	12 (66.7)	21 (84.0)
N1–N2	10 (11.8)	–	6 (33.3)	4 (16.0)
Leibovich score				
Low risk		30		7
Intermediate risk		12		12
High risk		–		6
Death [yes/no (%)]	25/60 (29.4/70.6)	3/39 (7.1/92.9)	14/4 (77.8/22.2)	8/17 (32.0/68.0)
Follow-up time, months [mean/median (range)]	66/72 (0–134)	82/79 (0–126)	22/13 (0–92)	70/72 (10–134)
Time to metastasis, months [mean/median (range)]	50/54 (0–126)	–	0/0	31/27 (2–73)

ccRCC clear cell renal cell carcinoma, *miRNA* microRNA

3p, miR-30c-5p, miR-30c-2-3p, miR-139-5p, miR-144-5p, miR-204-5p, and miR-451a) were selected for further expression analyses.

In the whole cohort (n = 108), a significantly decreased expression in metastatic tumors could be demonstrated for eight of the nine miRNAs (Table 2, Fig. 1). Downregulation of the remaining miRNA (miR-30c-5p) missed the level of significance (p = 5.9E−02). Furthermore, the expression levels of five miRNAs (miR-30a-3p, miR-30e-3p, miR-30c-2-3p, miR-10b-5p, and miR-144-5p) were significantly different in metastatic tumors compared with nonmalignant kidney tissue. The strong correlation to the metastatic risk of clinically localized tumors (M0) was verified for the nine miRNAs (area under the curve 0.654–0.727; $p \leq$ 1.0E−02). In addition, we found significant correlations of low expression levels of these nine miRNAs to high nuclear grading, as well as of seven miRNAs (miR-10b-5p, miR-30e-3p, miR-30c-2-3p, miR-139-5p, miR-144-5p, miR-204-5p, and miR-451a) to high T category (electronic supplementary Table S2).

In order to predict the metastatic risk more accurately, we investigated miRNA combinations with respect to the highest prognostic score of metastatic disease. Due to the limited amount of FFPE tissue, 85 tumor samples were available for these analyses. Thereby, we defined a 4-miRNA signature by combination of miR-30a-3p, miR-30c-5p, miR-139-5p, and miR-144-5p as the most powerful prognosticator. We performed an exhaustive feature selection, i.e. we tested all possible miRNA combinations from nine to two miRNAs. This revealed that the best performance could be reached with the four chosen miRNAs. Although miR-30c-5p did not reach significance as a single miRNA, it added complementary information. The accuracy of this prognostic model was 86%, with 91% sensitivity and 81% specificity, to predict metastases (synchronous and metachronous metastases). When measuring the performance of our model using five-times-repeated tenfold cross-validation, the overall accuracy of this model was still 77%, with 78% sensitivity and 76% specificity.

In univariate analysis, not only the 4-miRNA model but also tumor size, T category, grading, and nodal status were significantly associated with metastasis (Table 3a). Only the 4-miRNA model, nodal status, and T category could be confirmed as independent prognostic markers to predict the metastatic risk by multivariate testing (Table 3a). Thereby, the 4-miRNA model (hazard ratio [HR] 12.402, 95% confidence interval [CI] 3.586–42.893; p = 7.0E−05) remained a stronger predictor of metastasis-free survival than nodal status (HR 7.888, 95% CI 1.968–31.609; p = 3.5E−03) and T category (HR 1.950, 95% CI 1.270–2.993; p = 2.2E−03). Further extension of this 4-miRNA model using clinicopathological features did not improve the statistical power (data not shown). Univariate and multivariate comparison of the 4-miRNA model and the Leibovich score demonstrated the benefit of the molecular score and verified it as an independent score (Table 3b). Calculating the C-indices of both prognostic scores offered higher accuracy of the 4-miRNA model (C-index 0.86, p = 1.1E−06) compared with the Leibovich score (C-index 0.74, p = 1.0E−3). Kaplan–Meier curves showed an association between this 4-miRNA model and metastasis-free survival (p = 1.2E−09) (Fig. 2a), as well as with overall survival (p = 1.1E−04) (Fig. 2b).

In our cohort, 67 patients were diagnosed with clinically localized ccRCC. Of these, 37 tumors were diagnosed as low risk, 24 as intermediate risk, and 6 as high risk ccRCC

TABLE 2 Results of the whole cohort: expression differences of nine miRNAs in metastatic ccRCC versus nonmetastatic ccRCC versus nonmalignant kidney tissues of nephrectomy specimens

Comparison	miR-10b-5p	miR-30a-3p	miR-30c-2-3p	miR-30c-5p	miR-30e-3p	miR-139-5p	miR-144-5p	miR-204-5p	miR-451a
Nonmetastatic ccRCC versus metastatic ccRCC									
Fold change	**0.762**	**0.620**	**0.513**	0.779	**0.687**	**0.410**	**0.507**	**0.226**	**0.547**
p value	**1.0E−02**	**2.1E−03**	**6.0E−04**	5.9E−02	**1.3E−03**	**7.7E−05**	**5.4E−04**	**1.1E−03**	**3.6E−03**
Nonmalignant kidney tissue versus metastatic ccRCC									
Fold change	**0.443**	**0.349**	**0.371**	0.824	**0.651**	0.564	**1.848**	0.296	1.943
p value	**7.4E−06**	**3.5E−05**	**7.2E−05**	4.1E−01	**1.4E−02**	7.0E−02	**2.1E−02**	5.9E−02	5.2E−02
Nonmalignant kidney tissue versus nonmetastatic ccRCC									
Fold change	**0.581**	**0.563**	0.722	1.058	0.949	1.378	**3.643**	1.308	**3.551**
p value	**2.1E−03**	**3.1E−04**	1.0E−01	3.9E−01	6.4E−01	2.6E−01	**6.2E−07**	2.8E−01	**5.8E−06**

Significant p values ($p \leq$ 5.0E−2) are highlighted in bold along with fold change values

miRNA microRNA, *ccRCC* clear cell renal cell carcinoma

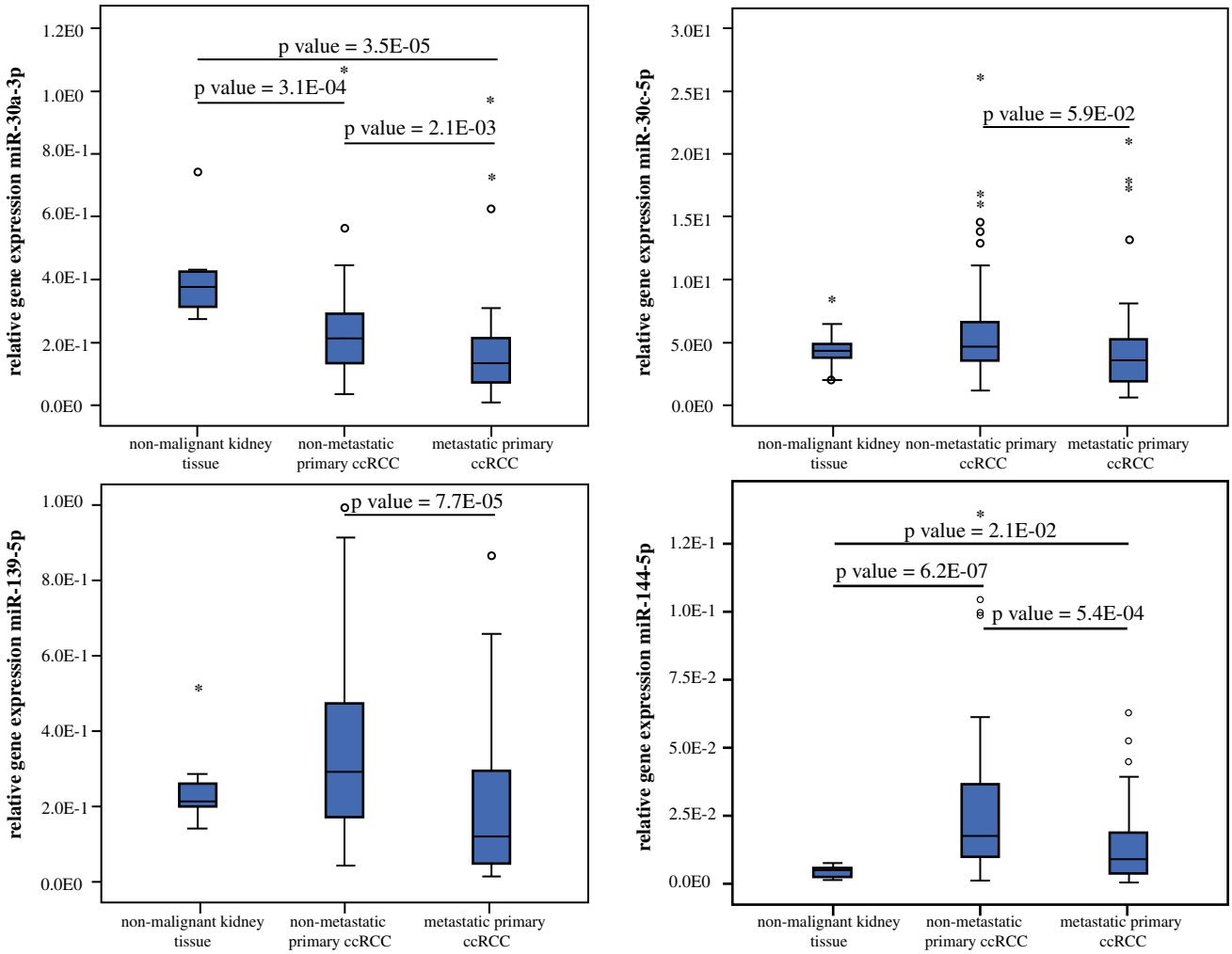

FIG. 1 Box plots of relative miRNA expression differences of metastatic ccRCC versus nonmetastatic ccRCC and nonmalignant kidney tissue. The Mann–Whitney U test defined significant differences as $p \leq 5.0E{-}2$. *miRNA* microRNA, *ccRCC* clear cell renal cell carcinoma

based on the Leibovich score (Table 1). From those, 19%, 50%, and 100% of patients, respectively, developed metastases during the postoperative disease course.

By applying the 4-miRNA model to the tumors with a low-risk Leibovich score ($n = 37$), five of seven patients with metachronous metastases, as well as 25 of 30 non-metastatic ccRCCs, were correctly classified. Univariate and multivariate analysis revealed the 4-miRNA model as the only independent prognostic factor for metastatic risk in low-risk tumors, with an HR of 7.887 (95% CI 1.523–40.847; $p = 1.4E{-}02$) (Table 3c). Kaplan–Meier analyses confirmed the high predictive value of the 4-miRA model for metastasis-free survival ($p = 3.4E{-}03$) (electronic supplementary Fig. S1a).

In intermediate-risk ccRCC patients ($n = 24$), 11 of 12 metachronously metastatic patients and 11 of 12 non-metastatic patients were correctly assigned using the 4-miRNA model. Multivariate analyses verified the 4-miRNA model as the only independent prognostic factor (HR 23.080, 95% CI 2.857–186.809; $p = 3.3E{-}03$) to identify metastatic and nonmetastatic tumors (Table 3d). Kaplan–Meier analyses further confirmed these results ($p = 4.4E{-}05$) (electronic supplementary Fig. S1b).

In the high-risk Leibovich score group, all patients developed metastases and were correctly assigned using our 4-miRNA model.

DISCUSSION

miRNAs have been demonstrated to be potent prognostic biomarkers associated with metastatic risk and survival in a variety of cancers, including ccRCC;[16–24] however, these miRNAs have not been implemented in current prognostic scores due to a lack of independent validations of their respective accuracy. In addition, dissemination and metastases formation is a multistep process, depending on multiple biological characteristics of each primary tumor. Thus, a biomarker profile reflecting the

TABLE 3 Univariate and multivariate Cox regression analyses of clinicopathological features, Leibovich score, and 4-miRNA model to metastasis-free survival of ccRCC patients with clinically localized tumors

Parameters	Univariate		Multivariate	
	p value	HR (95% CI)	*p* value	HR (95% CI)
(a) All clinically localized tumors (n = 67): 4-miRNA model versus clinicopathological features				
Age	NS		NS	
Sex	NS		NS	
Tumor size (cm)	**1.8E−05**	**1.388 (1.195–1.611)**	NS	
T category	**2.8E−04**	**2.070 (1.399–3.064)**	**2.2E−03**	**1.950 (1.270–2.993)**
Grading	**1.1E−03**	**3.968 (1.730–9.102)**	NS	
Nodal status	**2.3E−04**	**8.474 (2.718–26.423)**	**3.5E−03**	**7.888 (1.968–31.609)**
4-miRNA model	**5.6E−06**	**16.711 (4.958–56.321)**	**7.0E−05**	**12.402 (3.586–42.893)**
(b) All clinically localized tumors (n = 67): 4-miRNA model versus Leibovich score				
Leibovich score	**1.4E−05**	**3.632 (2.032–6.491)**	**9.3E−03**	**2.088 (1.199–3.636)**
4-miRNA model	**5.6E−06**	**16.711 (4.958–56.321)**	**6.7E−05**	**12.678 (3.636–44.207)**
(c) Clinically localized tumors with low-risk Leibovich score (n = 37): 4-miRNA model versus clinicopathological features				
Age	NS		NS	
Sex	NS		NS	
Tumor size (cm)	NS		NS	
Grading	NS		NS	
Nodal status	**2.4E−02**	**15.993 (1.450–176.405)**	NS	
4-miRNA model	**1.4E−02**	**7.887 (1.523–40.847)**	**1.4E−02**	**7.887 (1.523–40.847)**
(d) Clinically localized tumors with intermediate-risk Leibovich score (n = 24): 4-miRNA model versus clinicopathological features				
Age	NS		NS	
Sex	NS		NS	
Tumor size (cm)	**1.5E−02**	**1.332 (1.058–1.677)**	NS	
Grading	NS		NS	
Nodal status	NS		NS	
4-miRNA model	**3.3E−03**	**23.080 (2.851–186.809)**	**3.3E−03**	**23.080 (2.851–186.809)**

Significant *p* values ($p \leq 5.0E{-}2$) are highlighted in bold

Analyses were performed in (a, b) all tumors, (c) low-risk Leibovich score including category 0–2 tumors, and (d) intermediate-risk Leibovich score tumors including category 3–5 tumors

miRNA microRNA, *ccRCC* clear cell renal cell carcinoma, *HR* hazard ratio, *CI* confidence interval, *NS* nonsignificant

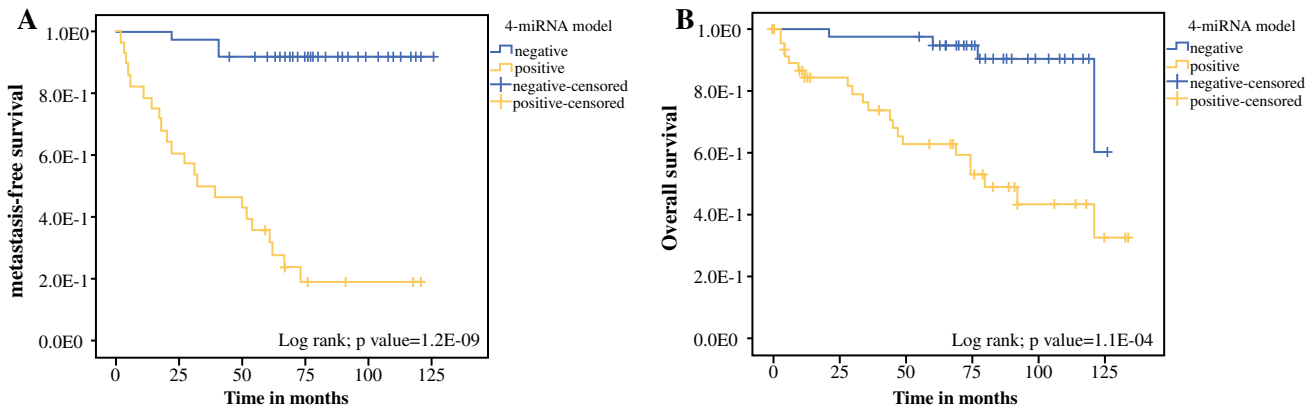

FIG. 2 Kaplan–Meier curves depicting **a** metastasis-free survival of clinically localized ccRCC and **b** overall survival of patients with ccRCC, stratified by positive versus negative 4-miRNA model. Log-rank test defined significant differences as $p \leq 5.0E{-}2$. *miRNA* microRNA, *ccRCC* clear cell renal cell carcinoma

metastatic potential of the tumor is superior compared with single biomarkers. We previously reported several miRNAs as being associated with metastatic risk using two independent sample cohorts.[14, 15] In the current study, we validated these miRNAs in a third independent patient cohort and successfully established a 4-miRNA model as an independent prognostic score with high accuracy of predicting the individual metastatic risk based on FFPE samples of primary tumors.

We validated nine previously described metastasis-associated miRNAs as the most promising marker candidates for aggressive ccRCC. Furthermore, lower miRNA expression was significantly correlated with higher T category and nuclear grading, indicating their strong association with aggressive phenotypes.

We established a 4-miRNA model (miR-30a-3p, miR-30c-5p, miR-139-5p, and miR-144-5p) as a promising tool for identifying tumors with high metastatic risk, using the random forest classifier. Since the model was trained on the complete dataset, the estimation of its performance on the same set would overestimate the prediction strength of the model on novel data. Therefore, we applied cross-validation to estimate the corresponding performance on novel data. In addition, we repeated the cross-validation to limit biases towards specific data splits on which our model might perform extremely well or poorly, which can happen easily when the number of patients is not very high. We chose a random forest classifier since it is well-suited for small datasets due to its ability to prioritize a lower variance over a smaller bias. We are aware that miR-144-5p is dysregulated in body fluids and tissue biopsies of many diseases, but, in combination with the three other miRNAs, the required specificity is likely reached.[25] The strong independent significance of the 4-miRNA model has been corroborated in multivariate analyses. The high prognostic value of this 4-miRNA model could be proven in those 67 patients with clinically localized (M0) tumors, predicting the development of distant metastases with high accuracy. Compared with clinicopathological factors, this miRNA model was the best independent predictor for risk stratification. Nodal status and T category as further independent factors showed lower HRs and weaker p values. The combination of molecular and clinical parameters did not improve the statistical power of our model. In addition, the 4-miRNA model is significantly correlated with overall survival, underlining its strong prognostic power.

We also compared the 4-miRNA model with the clinical prognostic Leibovich score, which defines low-, intermediate- and high-risk patients. Both prognostic models showed significant association with metastasis-free survival, but the 4-miRNA model was the stronger independent prognostic parameter. Even within low-risk patients, a prognostic group that is rarely associated with metastatic events, several patients developed metastases. The majority of those missed cases could be detected by our 4-miRNA model. Thus, this 4-miRNA model can predict the risk of metastasis in a more sensitive and individual manner. It is suitable for the selection of patients for adjuvant therapies based on biological determinants of metastasis rather than on clinicopathological features alone. The most recently published adjuvant therapy trials (ASSURE, S-TRAC, PROTECT) failed to show an overall survival benefit in patients with high-risk disease receiving adjuvant treatment with sunitinib, sorafenib, or pazopanib.[26–28] In these trials, the selection of high-risk patients was based on standard prognostic parameters such as T category, nodal status, and nuclear grading. T category and nuclear grading were not significant in our multivariate analyses, in contrary to the 4-miRNA model. Thus, future clinical trials should investigate whether molecular parameters, such as this miRNA model, can select patients for adjuvant strategies more accurately and can therefore increase its therapeutic effect.

Several studies have recently been published supporting the prognostic value of miRNA panels in ccRCC patients. Two miRNA signatures (miR-10b-5p, miR-139-5p, miR-130b, miR-199b-5p, and miR-21-5p, miR-142-5p, miR-194-5p) have been reported to predict the metastatic potential of primary tumors.[29,30] Another miRNA panel using miR-10a-5p and miR-223-3p discriminating non-metastatic against metastatic ccRCCs could be validated on biopsy specimens, although the predictive value was lower.[31] These studies support our hypothesis that miRNA panels outperform single miRNAs with regard to prognostic impact; however, the miRNA panels have not been tested against clinical scores and therefore the additional prognostic value was not proven, as in our study.

The robustness of miRNAs is a general advantage compared with messenger RNAs (mRNAs) that are more sensitive against degradation. Further studies are required to compare both miRNA and published mRNA signatures.[32]

This study is limited by its retrospective approach. In order to evaluate the clinical applicability of the 4-miRNA model with regard to the prognostic value and patient selection for continuous monitoring or adjuvant therapy, we are planning a prospective validation. Furthermore, intratumoral heterogeneity of molecular markers is still under debate.[33] However, the validation of these four miRNAs in three independent cohorts using only one tumor area argues against decisive tumor heterogeneity.

The functional role of the four miRNAs in metastasis, and possible targets, have been identified, at least in part, by both other groups and our own group (miR-139-5p,[34] miR-144-5p,[35] miR-30 family,[15,36,37] and unpublished data).

CONCLUSIONS

We established a prognostic 4-miRNA model based on the biological determinants of metastasis, which defines individual metastatic risk more accurately than currently used clinicopathological features or prognostic scores. By using this molecular model, ccRCC patients can be stratified more individually into high- and low-risk groups. The model should therefore facilitate risk-adapted follow-up and selection of patients suitable and informative for adjuvant treatment strategies after resection of the primary tumor.

ACKNOWLEDGMENT The authors thank Prof. Dr. Carsten Ohlmann and Dr. Johannes Linxweiler for critically reading this paper.

FUNDING This study was supported by a grant from the Wilhelm-Sander-Stiftung, Germany (Grant Number 2014.0007.1), and additionally supported by the Robert Bosch Foundation.

CONFLICT OF INTEREST Joana Heinzelmann, Madeleine Arndt, Ramona Pleyers, Tobias Fehlmann, Sebastian Hoelters, Philip Zeuschner, Alexander Vogt, Alexey Pryalukhin, Elke Schaeffeler, Rainer M. Bohle, Mieczyslaw Gajda, Martin Janssen, Michael Stoeckle, and Kerstin Junker have no commercial interests in the subject matter of this study.

REFERENCES

1. Cohen HT, McGovern FJ. Renal-cell carcinoma. *N Engl J Med*. 2005;353:2477–90.
2. Dabestani S, Thorstenson A, Lindblad P, Harmenberg U, Ljungberg B, Lundstam S. Renal cell carcinoma recurrences and metastases in primary non-metastatic patients: a population-based study. *World J Urol*. 2016;34:1081–1086.
3. Ljungberg B, Bensalah K, Canfield S, et al. EAU guidelines on renal cell carcinoma: 2014 update. *Eur Urol*. 2015;67:913–24.
4. Leibovich BC, Blute ML, Cheville JC, et al. Prediction of progression after radical nephrectomy for patients with clear cell renal cell carcinoma: a stratification tool for prospective clinical trials. *Cancer*. 2003;97:1663–1671.
5. Youssef RF, Cost NG, Darwish OM, Margulis V. Prognostic markers in renal cell carcinoma: a focus on the 'mammalian target of rapamycin' pathway. *Arab J Urol*. 2012;10:110–117.
6. van Vlodrop IJH, Joosten SC, De Meyer T, et al. A four-gene promoter methylation marker panel consisting of GREM1, NEURL, LAD1, and NEFH predicts survival of clear cell renal cell cancer patients. *Clin Cancer Res*. 2017;23:2006–2018.
7. Buttner F, Winter S, Rausch S, et al. Survival prediction of clear cell renal cell carcinoma based on gene expression similarity to the proximal tubule of the nephron. *Eur Urol*. 2015;68:1016–20.
8. Rini B, Goddard A, Knezevic D, et al. A 16-gene assay to predict recurrence after surgery in localised renal cell carcinoma: development and validation studies. *Lancet Oncol*. 2015;16:676–85.
9. Brooks SA, Brannon AR, Parker JS, et al. ClearCode34: a prognostic risk predictor for localized clear cell renal cell carcinoma. *Eur Urol*. 2014;66:77–84.
10. Morgan TM, Mehra R, Tiemeny P, et al. A multigene signature based on cell cycle proliferation improves prediction of mortality within 5 yr of radical nephrectomy for renal cell carcinoma. *Eur Urol*. 2018;73:763–769.
11. Sanjmyatav J, Hauke S, Gajda M, et al. Establishment of a multicolour fluorescence in situ hybridisation-based assay for subtyping of renal cell tumours. *Eur Urol*. 2013;64:689–91.
12. Youssef YM, White NMA, Grigull J, et al. Accurate molecular classification of kidney cancer subtypes using microRNA signature. *Eur Urol*. 2011;59:721–730.
13. Cho WC. MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets for therapy. *Int J Biochem Cell Biol*. 2010;42:1273–81.
14. Heinzelmann J, Henning B, Sanjmyatav J, et al. Specific miRNA signatures are associated with metastasis and poor prognosis in clear cell renal cell carcinoma. *World J Urol*. 2011;29:367–73.
15. Heinzelmann J, Unrein A, Wickmann U, et al. MicroRNAs with prognostic potential for metastasis in clear cell renal cell carcinoma: a comparison of primary tumors and distant metastases. *Ann Surg Oncol*. 2014;21:1046–54.
16. Fu Q, Liu Z, Pan D, et al. Tumor miR-125b predicts recurrence and survival of patients with clear-cell renal cell carcinoma after surgical resection. *Cancer Sci*. 2014;105:1427–34.
17. Slaby O, Redova M, Poprach A, et al. Identification of MicroRNAs associated with early relapse after nephrectomy in renal cell carcinoma patients. *Genes Chromosomes Cancer*. 2012;51:707–16.
18. Khella HW, Scorilas A, Mozes R, et al. Low expression of miR-126 is a prognostic marker for metastatic clear cell renal cell carcinoma. *Am J Pathol*. 2015;185:693–703.
19. Samaan S, Khella HW, Girgis A, et al. miR-210 is a prognostic marker in clear cell renal cell carcinoma. *J Mol Diagn*. 2015;17:136–44.
20. Fritz HK, Lindgren D, Ljungberg B, Axelson H, Dahlback B. The miR(21/10b) ratio as a prognostic marker in clear cell renal cell carcinoma. *Eur J Cancer*. 2014;50:1758–65.
21. Faragalla H, Youssef YM, Scorilas A, et al. The clinical utility of miR-21 as a diagnostic and prognostic marker for renal cell carcinoma. *J Mol Diagn*. 2012;14:385–92.
22. Zaman MS, Shahryari V, Deng G, et al. Up-regulation of microRNA-21 correlates with lower kidney cancer survival. *PLoS ONE*. 2012;7:e31060.
23. Teixeira AL, Ferreira M, Silva J, et al. Higher circulating expression levels of miR-221 associated with poor overall survival in renal cell carcinoma patients. *Tumour Biol*. 2014;35:4057–66.
24. Petillo D, Kort EJ, Anema J, Furge KA, Yang XJ, Teh BT. MicroRNA profiling of human kidney cancer subtypes. *Int J Oncol*. 2009;35:109–14.
25. Keller A, Leidinger P, Vogel B, et al. miRNAs can be generally associated with human pathologies as exemplified for miR-144. *BMC Med*. 2014;12:224.
26. Haas NB, Manola J, Uzzo RG, et al. Adjuvant sunitinib or sorafenib for high-risk, non-metastatic renal-cell carcinoma (ECOG-ACRIN E2805): a double-blind, placebo-controlled, randomised, phase 3 trial. *Lancet*. 2016;387:2008–16.
27. Ravaud A, Motzer RJ, Pandha HS, et al. Adjuvant sunitinib in high-risk renal-cell carcinoma after nephrectomy. *N Engl J Med*. 2016;375:2246–2254.
28. Motzer RJ, Haas NB, Donskov F, et al. Randomized phase III trial of adjuvant pazopanib versus placebo after nephrectomy in patients with locally advanced renal cell carcinoma (RCC) (PROTECT). *J Clin Oncol*. 2017;35:3916–3923.
29. Wu X, Weng L, Li X, et al. Identification of a 4-microRNA signature for clear cell renal cell carcinoma metastasis and prognosis. *PLoS ONE*. 2012;7:e35661.

30. Lokeshwar SD, Talukder A, Yates TJ, et al. Molecular characterization of renal cell carcinoma: a potential three-microRNA prognostic signature. *Cancer Epidemiol Biomark*. 2018;27:464–472.
31. Kowalik CG, Palmer DA, Sullivan TB, et al. Profiling microRNA from nephrectomy and biopsy specimens: predictors of progression and survival in clear cell renal cell carcinoma. *BJU Int*. 2017;120:428–440.
32. Hakimi AA, Voss MH. Genomic classifiers in renal cell carcinoma. *Eur Urol*. 2018;73:770–771.
33. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883–892.
34. Huang LL, Huang LW, Wang L, Tong BD, Wei Q, Ding XS. Potential role of miR-139-5p in cancer diagnosis, prognosis and therapy. *Oncol Lett*. 2017;14:1215–1222.
35. Yamada Y, Arai T, Kojima S, et al. Regulation of antitumor miR-144-5p targets oncogenes: direct regulation of syndecan-3 and its clinical significance. *Cancer Sci*. 2018;109:2919–2936.
36. Wang D, Zhu C, Zhang Y, et al. MicroRNA-30e-3p inhibits cell invasion and migration in clear cell renal cell carcinoma by targeting Snail1. *Oncol Lett*. 2017;13:2053–2058.
37. Mathew LK, Lee SS, Skuli N, et al. Restricted expression of miR-30c-2-3p and miR-30a-3p in clear cell renal cell carcinomas enhances HIF2alpha activity. *Cancer Discov*. 2014;4:53–60.