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Diagnostic and prognostic potential of differentially expressed miRNAs between metastatic and non-metastatic renal cell carcinoma at the time of nephrectomy

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ABSTRACT

Background: MicroRNAs are promising diagnostic and prognostic biomarkers in oncology. We aimed to evaluate the prognostic potential of selected microRNAs in primary clear cell renal cell carcinomas (ccRCC) as predictors of tumor recurrence after radical nephrectomy.
Methods: miR-122, miR-141, miR-155, miR-184, miR-200c, miR-210, miR-224, and miR-514, validated as differentially expressed in a previous study, were measured by RT-PCR in matched malignant and non-malignant tumor samples after nephrectomy from 111 patients (89 without, 22 with metastases) and clinicopathological and outcome data were collected. Non-parametric statistical tests, receiver-operating characteristics, Kaplan–Meier-, and univariate as well as multivariate Cox regression analyses were performed.
Results: Downregulation of miR-141/-184/-200c/-514 and upregulation of miR-122/-155/-210/-224 were not different between samples of non-metastatic and metastatic tumors except for miR-122 and miR-514. miR-514 was further downregulated in metastatic compared with non-metastatic tumors while the upregulation of miR-122 was significantly reduced in metastatic carcinomas. All miRNAs were suitable to discriminate malignant from non-malignant tissue. miR-122 and miR-514 were significantly related to the recurrence risk but only miR-514 provided independent prognostic information in the final model including relevant clinicopathological variables.
Conclusions: MiR-122 and miR-514 play a role in tumor recurrence after nephrectomy. Expression of miR-514 was particularly downregulated in primary metastatic tumor and those that recur and might be a suitable adjunct marker for predicting tumor recurrence.

1. Introduction

The integration of molecular testing in therapy planning of solid tumors including breast, colon, and lung cancer or malignant melanoma is currently transforming clinical practice. In contrast, similar efforts for renal cell carcinoma (RCC) have not been successful so far [1]. RCC has one of the highest cancer-specific mortality rates and a permanently increasing incidence of 2.5% over the years representing 3–5% of all adult malignancies [2]. Prognostic information for RCC patients is still based only on clinicopathological and imaging data. Prognostic models developed with the aid of these data cannot compensate for their inherent limited information [3]. Thus, there is an urgent need for new molecular markers that can alone or in combination with the traditional data fulfill the demands for improving diagnosis, risk stratification, and prediction of therapeutic response for RCC patients [4].

MicroRNAs (miRNAs) have emerged as important cancer biomarkers. miRNAs are small (approximately 20–22 nucleotides), non-protein coding transcripts some of which are strongly involved in carcinogenesis. Differential expression data of miRNAs and their role as regulators in signal transduction and metastasis have been compiled for urological malignancies [4–7]. Several studies have reported on the diagnostic and prognostic impacts of miRNA expression for RCC [8–15]. However, only three studies really presented prognostic data but without considering the multifactorial nature of the actual miRNA changes [16–18]. In this respect, clear information is missing and is urgently needed based on multivariate analyses.

In a previous microarray profiling study of ccRCC samples, we found miR-122a, miR-155, miR-210, and miR-224 strongly upregulated and miR-141, miR-184, miR-200c, and miR-514 downregulated [10].

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However, the prognostic potential of these miRNAs has not yet been elucidated. Therefore, the aims of the present study were (a) to confirm the differential expression of these miRNAs in a new cohort of ccRCC patients as part of an external validation and (b) to evaluate the potential of these miRNAs as prognostic markers, both alone and in combination with clinicopathological data, for tumor recurrence following radical nephrectomy.

2. Materials and methods

2.1. Patients and tissue samples

The local Ethics committee approved the study and informed patient consent was obtained. Tissue from the primary tumor and matched normal adjacent tissue were collected after radical nephrectomy at the University Hospital Charité between 2005 and 2009. Tissue samples were either snap frozen in liquid nitrogen or immersed in the stabilizing RNA Later solution immediately after surgery and were stored at −80 °C. Samples collected from ccRCC patients were selected according to the availability of cryo-preserved tissue. The study also included 18 samples of the preceding profiling study [10] and eight bone metastatic samples of the reference gene study [19]. For each patient, clinical and pathologic information was gathered (Table 1). The tumors were classified according to the 2002 TNM classification [20] and the 2004 WHO criteria at the Department of Pathology of the Charité (GK, AE, EK).

2.2. RNA extraction and quantitative RT-PCR

Methods of RNA isolation and quantitative RT-PCR (RT-qPCR) were previously described [10,19,21] and were performed according to the MIQE guidelines [22]. Briefly, frozen histologic sections, stained with hematoxylin/eosin, were prepared from the collected tissue samples and only samples with at least 80% tumor cells were used. RNA was extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany). RNA extracts had a median RNA integrity number of 7.8 (range: 6.1–9.5). RT-qPCRs were performed using TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol on the Light-Cycler 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany) [10,19,21]. The miScript PCR System (Qiagen) was additionally used for the determination of miR-514. The characteristics of all assays including additional methodical details and method comparisons of miR-122 and miR-514 using assays according to the mirBase 9.2 and 18 were compiled in Supplementary Data.

Table 1
Clinical and pathologic characteristics of the patients evaluated in the study.

Characteristic	Primary ccRCC, non-metastatic	Primary ccRCC, metastatic	*p*-Value
Patients, no.	89	22	
Gender, male/female	64/25	13/9	0.303[a]
Age, yrs, median (range)	65 (39–82)	62 (40–75)	0.238[b]
Pathological stage, no. (%)			
pT1a	29 (33)	1 (5)	0.002[a]
pT1b	22 (25)	2 (9)	
pT2	4 (5)	0 (0)	
pT3a	16 (18)	8 (32)	
pT3b	15 (17)	7 (32)	
pT3c	2 (2)	1 (5)	
pT4	1 (1)	3 (14)	
Grade, n (%)			
G1	12 (14)	0 (0)	<0.0001[a]
G2	69 (78)	8 (36)	
G3	6 (7)	12 (55)	
G4	2 (2)	2 (9)	
Surgical margins, n (%)			
R0	82 (92)	13 (59)	0.0004[a]
R1	6 (7)	8 (36)	
R2	1 (2)	1 (5)	
Tumor size, mm, median (range)	50 (13–220)	90 (30–180)	<0.0001[b]
Patients followed, n (%)	87 (100)	21 (100)	
Metastasis at followup	17 (20)		
Death at followup	10 (12)	17 (81)	<0.0001[b]
Follow-up, mo, median (range)			
Overall	33.8 (2.0–77.1)	9.4 (1.2–54.8)	<0.0001[b]
No recurrence	35.1 (2.0–77.1)		
Recurrence at	8.0 (3.4–44.8)		

Abbreviations: ccRCC: clear cell renal cell carcinoma.
[a] *p*-Value from Fisher's exact test or Chi-square test.
[b] *p*-Value from Mann–Whitney *U*-test.

2.3. Data analysis and statistical analysis

Data analysis concerning the correction of amplification efficiency, the compensation of differences between runs by normalizing with inter-plate calibrators, and the normalization of miRNA expression with the reference gene combination were performed with GenEX software (MultiD Analyses, Göteborg, Sweden) [23]. Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) using the bootstrapping module for internal validation as indicated, GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA, USA), and MedCalc 12.3.0.0 (MedCalc Software, Mariakerke, Belgium). Non-parametric tests (Mann–Whitney *U*-test, Spearman rank correlation) were used. Receiver operating characteristic (ROC) curves and logistic regression served to identify the best discriminating combinations of miRNAs and to calculate the percentage overall correct classifications. The Kaplan–Meier approach and Cox proportional hazard regression analysis were used for disease progression analyses. $p<0.05$ (two-sided) was considered statistically significant. GraphPad Statmate 2.0 was used for sample size determinations (Supplementary Data).

3. Results

3.1. Patient characteristics

A total of 111 patients with ccRCC either without (n = 89) or with metastasis (n = 22) at the time of surgery were enrolled; their clinicopathological characteristics are summarized in Table 1.

3.2. Expression of miRNAs in relation to clinicopathological features

The expression of the eight miRNAs was normalized to the reference gene panel of miR-28, miR-103, and miR-106a [19]. Age and sex did not correlate with their expression ($r_S = 0.014–0.177$, $p = 0.065–0.882$). In Fig. 1, the expression rates are presented as fold changes relative to the matched normal adjacent tissue. These data confirmed the up- and down-regulated miRNAs of our previous profiling study [10]. Of these eight miRNAs, only miR-122 and miR-514 differed in their expression in primary non-metastatic and metastatic tumors, showing in both cases a decreased expression in metastatic tumors. Method comparisons for these two miRNAs showed interchangeable results when measurements were performed either with assays based on miRNA sequences of the mirBase 9.2 used for the profiling study [10] or with those of the recent mirBase 18 in their corrected annotations (Supplementary Data).

To verify this progression-dependent expression of the two miRNAs, we measured their expression in eight metastatic bone samples from ccRCC patients. Here too, their fold changes, calculated in comparison with the median expression in normal adjacent tissue of the non-metastatic study group, were significantly lower than those in the non-metastatic tumors (miR-122: 7, 95% CI 0.9–27 vs. 34, 95% CI 17–50, $p = 0.040$; miR-514: −198, 95% CI −136 to −480 vs. −97, 95% CI −57 to −149, $p = 0.038$; Mann–Whitney *U*-test), but did not differ from the primary metastatic RCCs ($p = 0.887–0.661$).

These expression levels were also associated with the pT stage and grade. In the tumor specimens of stage pT3 + 4 or G3 + 4 compared to