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The miR$^{21/10b}$ Ratio As a Prognostic Marker in Clear Cell Renal Cell Carcinoma

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Abstract

**Purpose:** Clear Cell Renal Cell Carcinoma (ccRCC) is the most common type of cancer in the adult kidney, and the prognosis of metastatic ccRCC remains poor with high mortality. In ccRCC, microRNAs (miRs) differentially expressed in tumor tissue have been identified and have been proposed to predict prognosis. The purpose of this study was to evaluate candidate miR markers identified from analysis of The Cancer Genome Atlas (TCGA) datasets in a large RCC cohort and to elucidate whether a ratio of miRs provided additional prognostic information.

**Experimental Design:** Deep sequencing data from TCGA datasets were analyzed using biostatistical methods to identify candidate miRs that correlate with factors such as survival and stage of disease. Candidate miRs were analyzed by RT-qPCR in a cohort of 198 RCC tumors (ccRCC, n=152) and 50 normal kidney samples.

**Results:** Four candidate miRs (miR-10b, miR-21, miR-101, and miR-223) were selected from the TCGA analysis and analyzed in our cohort. Of these, miR-21 and miR-10b were differentially expressed in RCC subtypes and in ccRCC nuclear grades. Individually, the two miRs demonstrated a non-significant trend to correlate with survival. Importantly, the ratio of miR-21/miR10b (miR^{21/10b}) correlated significantly with disease severity and survival, a high miR^{21/10b} being associated with poor prognosis ($P = 0.0095$). In particular, the miR^{21/10b} was found to be an independent prognostic factor in metastasis-free patients ($P = 0.016; CI 1.201-5.736$).
Conclusions: We have shown that the miR$^{21/10b}$ ratio is an independent prognostic factor for M0 ccRCC patients, which could be useful to identify high-risk M0 patients who could benefit from increased surveillance.

Keywords: Biomarker, Cancer, Clear Cell Renal Cell Carcinoma, Kidney Cancer, microRNA, miRNA, Prognosis, RCC, Renal Cancer
**Introduction**

Renal Cell Carcinoma (RCC) is the ninth most common malignancy in Europe, accounting for approximately 2% of all cancer cases in adults(1). RCC is by far the most common cancer of the kidney, accounting for 85% of the kidney cancer cases(2), and is further classified into three major subtypes; clear cell RCC (ccRCC), papillary RCC (pRCC), chromophobe RCC (chRCC), along with several less common subtypes of RCC(3, 4). Clear Cell RCC accounts for approximately 75% of all RCCs(3, 4) and is often characterized by the appearance of a ‘clear’ cell cytoplasm due to accumulation of lipids in the tumor cells. Tumor-Node-Metastasis (TNM) stage is the most important prognostic variable(4), where patients are scored and staged based on tumor extent, lymph node involvement, and presence of distant metastasis. Furthermore, it has been shown that additional parameters such as the tumor size, the nuclear grade and the presence of necrosis provide additional information for risk assessment of patients. Postoperative prognostic systems and nomograms that combine independent prognostic factors have been developed and validated(5, 6). These nomograms aim to measure predictive accuracy by combining different variables including the TNM stage(7).

RCC in general respond poorly to chemotherapy(8), and radiotherapy(9). Surgery remains the only curative treatment(9). Early detection is of great importance for patient outcome, the 5-year survival for patients diagnosed with organ-confined disease is approximately 93%(10), whereas the prognosis of patients with distant metastasis remains poor with a 5-year survival of less than 10%(8). Around 6% of the patients with
RCC present with metastatic disease (10), and in addition another 30% of patients who undergo complete surgical resection of the localized tumor eventually develop distant metastasis (11). Because of the poor prognosis associated with metastasis in ccRCC, development of additional prognostic tools that can help identify patients that are likely to develop metastasis, or respond to treatment, would be of great value.

MicroRNAs (miRs) are short, non-coding, single-stranded RNA molecules, approximately 22 nucleotides long, that can bind to and act as post-transcriptional regulators of target mRNAs (12). It has been predicted that as much as 30% of the human genome is regulated by miRs (13), and each miR can regulate translation of hundreds of target mRNAs (14, 15). In RCC, several studies have been carried out with the aim to identify miRs that are differentially expressed and associated with patient outcome (16-22), although the greater part of these studies have been carried out in relatively small cohorts.

In a recent study by The Cancer Genome Atlas (TCGA) research network, a large ccRCC cohort was used to identify parameters with prognostic potential, including several miRs (23). We have conducted a study in a large and well-characterized ccRCC cohort, determining the expression of candidate miRs identified in the TCGA study, as well as their correlation with currently used prognostic factors and survival. Furthermore, we have employed the novel concept of a microRNA Index ratio (24), comprised of two of the candidate miRs, and demonstrate that miR\(^{21/10b}\) is an independent prognostic factor for patients having no metastasis at the time of diagnosis.
Materials and Methods

External data sets

Level 3 RNA-seq data containing normalized miR expression values were downloaded from The Cancer Genome Atlas (TCGA) data portal (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) by March 2013. The data comprised 284 ccRCCs analyzed on the Illumina Genome Analyzer (GA) platform and 217 ccRCCs analyzed on the Illumina HiSeq platform. Matched clinical follow up data for tumors were obtained from the TCGA data portal and deceased patients were considered as dead from renal cancer-related causes as defined in Hakimi et al(25). Cox proportional-hazard regression analysis was performed on logged normalized expression values on each miR within the two data sets using the Survival package in R (http://cran.r-project.org). The Benjamini & Hochberg method was used to correct for multiple testing.

Patient cohort information

The internal study cohort comprised samples from a total of 198 RCC patients. In addition, 50 histologically non-malignant kidney cortex tissue samples were included. Matched tumor and normal tissues were available from 45 patients. The institutional review board and the ethical committee of Umeå University approved the study, and each patient provided their informed consent. Samples were obtained at the time of nephrectomy, which was performed at the Department of Urology, Umeå University Hospital, between 1985 and 2003. Definitions of the data parameters and the design study have been described in detail elsewhere(26). The mean follow-up time for surviving
patients was 128.1 ± 57.9 months, n = 51. During follow-up, 46 patients were alive without any indications of disease, 5 were alive with disease, 100 did not survive their disease (disease-specific death) and 47 passed away of other causes.

**Tissue RNA extraction**

For RNA studies, tissue samples from patients were obtained immediately after nephrectomy, and the viable area of each tumor or non-malignant sample was used for total RNA extraction using TRIzol reagent (Ambion, Austin, TX, USA). The quality of the extracted RNA was evaluated using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), as well as agarose gel electrophoresis followed by ethidium bromide staining of 18S and 28S rRNA. Spectrophotometrical quantification of RNA concentrations was done at a wavelength of 260 nm on a DU640 spectrophotometer (Beckman Coulter, Brea, California, USA). All samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

**Reverse transcription and quantitative PCR (RT-qPCR)**

The mature miR expression levels were quantified using the TaqMan MicroRNA Assay protocol and reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s protocol with some minor changes. Expression of four miRs was measured; hsa-miR-10b, hsa-miR-21, hsa-miR-101 and hsa-miR-223. The Reverse Transcription (RT) step was run on a T100 thermal cycler (Bio-Rad Laboratories, Munich, Germany), and subsequent qPCR assays on a 7900HT Real-Time PCR System (Applied Biosystems). Along with each RT and qPCR reaction plate, negative controls
and inter-plate control samples were run. Quantification of the miR expression levels was done using the comparative $-\Delta \Delta Ct$ method. Ten candidate small non-coding RNAs (U47, RNU6B, RNU19, RNU24, RNU38B, RNU44, RNU48, RNU49, RNU58A, RNU66) were measured on 7 tumor samples and 7 normal kidney cortex samples. In accordance with analysis for suitable internal control RNAs using qbase+ GeNorm software (http://www.biogazelle.com/qbaseplus), the geometrical mean of U47, RNU44, and RNU48 was used for normalization as previously described(27).

**Statistical analysis**

The Mann-Whitney $U$ test was used for comparing expression levels in malignant and non-malignant samples, and the Wilcoxon test was used for pairwise comparison. For statistical analysis of expression levels between RCC subtypes, Kruskal-Wallis multiple comparison test was used. For survival analysis, the Kaplan-Meier method was used, and the Log-rank test was used for comparing cumulative survival. Multivariate analysis for independent prognostic variables was performed using Cox proportional hazard regression model, stepwise backward likelihood ratio method. The TCGA cohort was analyzed using Cox proportional-hazard regression, and the Benjamini & Hochberg method was used to correct for multiple testing. All the statistical tests were done using the SPSS software package (version 21, IBM), and all tests were two-sided with the significance level set to 0.05.
Results

Identification of candidate miRs using TCGA RNA-seq data.

To search for miRs that are associated with prognosis in ccRCC, two external RNA-seq expression data sets, comprising 284 and 217 ccRCCs, respectively, from The Cancer Genome Atlas (TCGA) data portal were examined. We employed Cox proportional-hazard ratio test on all miRs to find the most significant predictive miRs in the data (Table 1). For eight of the ten most significant miRs, increased expression was associated with a worsened prognosis, in terms of tumor grade and patient survival, whereas two were associated with a better prognosis.

MicroRNA expression levels in RCC. Based on the findings in TCGA RNA-seq data, four candidate miRs were selected for further analysis by RT-qPCR in our cohort. To select the candidate miRs, a list was compiled consisting of the top ten ranked miRs in the TCGA cohort using Cox proportional-hazard regression model (Table 1). Out of the ten miRs on the list, the four miRs with the highest relative expression were selected, in order to ensure that analysis by RT-qPCR was feasible. The expression of four candidate miRs, miR-10b, miR-21, miR-101, and miR-223, was determined using RT-qPCR in a large RCC cohort (n=198) comprised of a representative selection of RCC tumor subtypes (Table 2). For all candidate miRs, expression levels differed between normal kidney cortex and the RCC subtypes (Supplementary Fig. 1). In addition, pairwise analysis of the 35 matched ccRCC tumor and non-malignant kidney cortex tissue samples revealed that all of the miRs were differentially expressed in tumor tissue, miR-10b and
miR101 being downregulated, while miR-21 and miR-233 were found to be upregulated (Supplementary Fig. 2).

**miR-10b and miR-21 expression in relation to tumor nuclear grade and Tumor-Node-Metastasis (TNM) stage.** Expression of both miR-10b (Fig. 1A) and miR-21 (Fig. 1B) were found to be associated with tumor nuclear grade in ccRCC patients (n=152). For miR-10b, a decreased expression was observed in higher tumor grades, whereas the opposite was observed for miR-21. No differential expression was, however, seen for miR-101 or miR-223 with respect to tumor nuclear grade (data not shown). Furthermore, miR-10b expression was lower in the higher TNM stage (Fig. 1C), whereas expression levels of miR-21 (Fig. 1D), miR-101 and miR-223 were not significantly different between the TNM groups (data not shown). In contrast to what was recently shown in the TCGA RNA-seq data, none of the individual candidate miRs correlated significantly with survival for the whole ccRCC cohort, although a non-significant trend was observed for miR-10b (P=0.052, data not shown). However, in patients without metastasis at the time of diagnosis (M0), miR-10b correlated inversely with survival (P=0.012, data not shown), while for miR-21 there was a non-significant trend to positive correlation with survival (P=0.052, data not shown).

**The miR21/miR10b ratio is associated to tumor nuclear grade and TNM stage.** We hypothesized that a miR ratio could improve the usefulness of marker miRs in ccRCC, and therefore created a miR ratio for each individual sample defined as the expression ratio of logged normalized expression values between miR-21 and miR-10b (miR$_{21/10b}$).
Importantly, the miR$^{21/10b}$ showed a stronger association to both tumor nuclear grade (Fig. 1E) and TNM stage (Fig. 1F) than either of the two individual miRs alone. We also evaluated other ratio combinations, e.g. using all the four candidate miRs, but the inclusion of the two additional miRs did not result in improved prognostic value.

**High miR21/miR10b ratio associates with adverse outcome.** The strong association between miR$^{21/10b}$ and clinical-pathological parameters led us to investigate possible correlation to patient outcome. Indeed, miR$^{21/10b}$ was found to be lower in patients surviving ccRCC, than in those who did not survive (Fig. 2A). Furthermore, Kaplan-Meier analysis with the cut-off set at the miR$^{21/10b}$ median revealed that a low miR$^{21/10b}$ was associated with longer survival (Fig. 2B). The patients belonging to the group with low miR$^{21/10b}$ had a median survival of 172 months (CI: ±45.6 months), while patients in the high miR$^{21/10b}$ group had a median survival of 32 months (CI: ±6.1 months). Five-year survival was 56.6% and 37.9% for miR$^{21/10b}$-low and -high patients, respectively, and the 10-year survival remained at 56.6% for miR$^{21/10b}$-low patients, while the survival of miR$^{21/10b}$-high patients was decreased to 32.6%.

**miR$^{21/10b}$ provides independent prognostic information for M0 patients.**

Metastatic ccRCC is associated with poor prognosis, with a five-year survival of less than 10%, and in our cohort, all of the M1 patients died within 9 years of diagnosis and high/low miR$^{21/10b}$ had no prognostic value. This suggested that the prognostic potential of the miR$^{21/10b}$ would be of higher value for the M0 patient group, possibly providing a clinical tool to identify patients with a greater risk of eventually developing metastasis.
The miR$_{21/10b}$ did not differ between patients with or without metastasis (M1 vs. M0) at the time of diagnosis (Fig. 2C). The Kaplan-Meier analysis for the M0 patients revealed that those with low miR$_{21/10b}$ had longer disease-specific survival than those with high miR$_{21/10b}$ (Fig. 2D). The median survival was 223 months (CI: ±37.1 months) in the low miR$_{21/10b}$ M0 group, whereas the median survival was 94 months (CI: ±63.8 months) in the high miR$_{21/10b}$ M0 group. The 5-year survival was 84.2% for the low miR$_{21/10b}$ M0-group and 51.6% for the high miR$_{21/10b}$ group. Corresponding numbers after 10 years were 84.2% and 49.1%, respectively. Importantly, multivariate analysis for prognostic factors was performed according to the Cox proportional hazard regression model with miR$_{21/10b}$ cut-off set at the median, and revealed that for M0 patients, the miR$_{21/10b}$ is an independent prognostic factor (Table 3; $P=0.016$, CI 1.201-5.736) along with tumor diameter and TNM stage.
Discussion

In this report, a 2-miR ratio, $\text{miR}^{21/10b}$, was shown to be an independent prognostic factor for ccRCC patients without metastasis at the time of diagnosis. Although many studies have identified multiple miRs with potential prognostic or diagnostic use(28), the concept of the miR-ratio is relatively novel(24). The benefits of a ratio is that it eliminates the need of internal control RNAs, thereby reducing cost, and in addition it may amplify the impact of single miRs in terms of prognostic potential. Another practical advantage for the $\text{miR}^{21/10b}$ ratio is the high expression levels of both miR-21 and miR-10b in ccRCC, facilitating reliable RT-qPCR amplification in the clinical routine. The $\text{miR}^{21/10b}$ may be useful for patients presenting without metastasis, to identify those having poor prognosis and that could benefit from closer follow-up.

Even though the $\text{miR}^{21/10b}$ was a prognostic marker for patients without metastasis, the distribution of $\text{miR}^{21/10b}$ did not differ between M0 and M1, indicating that the $\text{miR}^{21/10b}$ is not predictive for the presence of metastasis. However, the strong association between a high $\text{miR}^{21/10b}$ and poor prognosis in M0 patients suggested that in the M0 patients, a high $\text{miR}^{21/10b}$ may increase the risk of metastasis or recurrence.

The molecular mechanisms explaining the discrepancy between predictive value for the $\text{miR}^{21/10b}$ in M1 and M0 patients are presently unknown. The biological implications of a high $\text{miR}^{21/10b}$ are not understood at present; the effects of miR-21 in ccRCC as a negative regulator of tumor suppressors have been studied(21, 29), while such studies
have not yet been carried out with respect to miR-10b in ccRCC. Expression of both miR-21 and miR-10b was found to be significantly different in tumor nuclear grades, indicating that these miRs are associated to tumor severity. Similarly, high miR-10b expression was significantly associated to decreased survival in M0 ccRCC patients, and high miR-21 expression demonstrated a non-significant trend to be associated with worse prognosis. Taken together, the association of the two miRs to both survival and tumor nuclear grade indicates that the miR ratio is biologically relevant and our results suggest that it provides important prognostic information and could possibly be incorporated into the available prognostic models and nomograms, such as the SSIGN algorithm (5), and the Leibovich score (6).

The miR-10b is generally considered to be an oncomiR, which regulates tumor suppressors and is upregulated in many cancers(30, 31). In contrast, we found that miR-10b is downregulated in ccRCC tumors and correlates inversely with survival in M0 patients. Although miR-10b has an expression profile in ccRCC that is opposite of that found in most other human cancers(30, 31), several previous RCC studies, including the TCGA project, have shown that miR-10b indeed is downregulated in RCC tumors(16, 17, 32). The mechanisms involved in downregulating the expression of miR-10b in ccRCC, and whether this contributes to tumorigenesis are presently unknown. Possibly, the effects of miR-10b are cell type-dependent(30).

miR-21 was one of the first oncomiRs to be identified, and it has since been confirmed to be upregulated in a great number of human cancers(33), including ccRCC(20, 21). In
RCC, increased expression of miR-21 is associated with increased proliferation and invasion, and decreased apoptosis (21, 29, 34). In addition, miR-21 has been indicated to correlate with disease-specific survival in RCC patients, however this study was carried out in a relatively small cohort (n=54) with five years of follow-up and without multivariate analysis (21). Similarly, other studies have been carried out to identify miRs that are associated with metastatic recurrence (22), or early relapse (16), however their independence as prognostic factor was not studied.

Interestingly, it has been shown that miR-21 expression is increased in patients with renal fibrosis (35). Furthermore, it has been demonstrated that IL-6 can induce miR-21 expression through STAT3, and that even transient miR-21 expression causes epigenetic reprogramming, activating an inflammatory positive feedback loop which seems to induce and maintain transformation in several tumor types (36).

**Conclusion**

In conclusion, we have identified four candidate miRs in ccRCC, and shown that a 2-microRNA ratio composed of miR-21/miR-10b, miR$^{21/10b}$, is an independent prognostic factor for M0 patients in ccRCC. As RT-qPCR is a readily available technique, determination of miR$^{21/10b}$ could possibly be useful in a clinical setting to identify high-risk M0 patients who could benefit from intensified post-operative surveillance.
Conflict of interest statement

None declared.

Acknowledgements

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References

Figure Captions

Figure 1. Relative expression of miR in ccRCC tumor grades and stages (n=152). Relative expression of A, miR-10b, and B, miR-21, in tumor nuclear grades, I+II versus III+IV. C, miR-10b, and D, miR-21 expression in TNM2002 tumor stage groups, I+II versus III+IV. Line represents median. E, miR ratio (miR$^{21/10b}$) in ccRCC tumor grades I+II versus III+IV. F, miR$^{21/10b}$ in TNM2002 stage groups I+II versus III+IV. Mann-Whitney U test. ****, p < 0.0001; ***, p < 0.001; **, p < 0.01; *, p < 0.05. The miR$^{21/10b}$ is defined as the ratio of miR-21 expression over miR-10b expression.

Figure 2. miR$^{21/10b}$ levels correlate with survival in ccRCC (n=152). A, miR$^{21/10b}$ levels in ccRCC tumors of surviving versus deceased patients. B, Kaplan-Meier patient survival analysis with miR$^{21/10b}$ divided into low (≤50th percentile) and high (>50th percentile) levels, respectively. C, miR$^{21/10b}$ in ccRCC patients without (M0) or with metastasis (M1) at time of diagnosis. D, Kaplan-Meier patient survival analysis in ccRCC tumors of patients without metastasis at diagnosis, with miR$^{21/10b}$ divided into low (≤50th percentile) and high (>50th percentile) levels, respectively. A, C; Mann-Whitney U test. **, p < 0.01. The miR$^{21/10b}$ is defined as the ratio of miR-21 expression over miR-10b expression.
Tables

Table 1. Candidate miRs identified in the TCGA dataset. P-value with respect to significance using Cox regression; HiSeq and GA represent the two different Deep Sequencing platforms used for data acquisition at the TCGA project. The Benjamini & Hochberg method was used to correct for multiple testing.

<table>
<thead>
<tr>
<th>miR</th>
<th>p-value (HiSeq)</th>
<th>p-value (GA)</th>
<th>Association</th>
<th>Mean log2 expression (HiSeq)</th>
<th>Mean log2 expression (GA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa.mir.130b</td>
<td>1.09E-06</td>
<td>2.08533E-05</td>
<td>Positive</td>
<td>3.26</td>
<td>3.69</td>
</tr>
<tr>
<td>hsa.mir.21</td>
<td>6.81E-05</td>
<td>0.000261411</td>
<td>Positive</td>
<td>16.53</td>
<td>17.20</td>
</tr>
<tr>
<td>hsa.mir.767</td>
<td>6.81E-05</td>
<td>0.0007038</td>
<td>Positive</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>hsa.mir.34c</td>
<td>9.42E-04</td>
<td>0.000074681</td>
<td>Positive</td>
<td>1.49</td>
<td>1.45</td>
</tr>
<tr>
<td>hsa.mir.101.1</td>
<td>1.03E-04</td>
<td>0.002225065</td>
<td>Negative</td>
<td>13.39</td>
<td>13.93</td>
</tr>
<tr>
<td>hsa.mir.105.2</td>
<td>4.91E-04</td>
<td>0.001582619</td>
<td>Positive</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>hsa.mir.223</td>
<td>2.35E-03</td>
<td>2.57408E-05</td>
<td>Positive</td>
<td>6.56</td>
<td>6.89</td>
</tr>
<tr>
<td>hsa.mir.105.1</td>
<td>4.26E-04</td>
<td>0.001944834</td>
<td>Positive</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>hsa.mir.153.2</td>
<td>5.46E-05</td>
<td>0.004062109</td>
<td>Positive</td>
<td>2.61</td>
<td>3.08</td>
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<tr>
<td>hsa.mir.10b</td>
<td>1.05E-03</td>
<td>0.00134504</td>
<td>Negative</td>
<td>17.38</td>
<td>17.10</td>
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</table>
Table 2. Cohort clinical characteristics with regards to RCC tumor subtype.

<table>
<thead>
<tr>
<th>Variable</th>
<th>pRCC</th>
<th>ccRCC</th>
<th>chRCC</th>
<th>Oncocytoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>27</td>
<td>152</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Patient sex, male/female</td>
<td>17/10</td>
<td>87/65</td>
<td>4/7</td>
<td>4/4</td>
</tr>
<tr>
<td>Patient age, range years</td>
<td>25-82</td>
<td>36-85</td>
<td>36-80</td>
<td>50-80</td>
</tr>
<tr>
<td>TNM stage, I+II/III/IV</td>
<td>14/7/6</td>
<td>66/38/48</td>
<td>5/4/2</td>
<td>-</td>
</tr>
<tr>
<td>Metastasis status, M0/M1</td>
<td>21/6</td>
<td>105/47</td>
<td>9/2</td>
<td>-</td>
</tr>
<tr>
<td>Nuclear grade, 1/2/3/4</td>
<td>4/9/11/3</td>
<td>8/33/77/34</td>
<td>0/2/8/1</td>
<td>-</td>
</tr>
<tr>
<td>Tumor size, mm range</td>
<td>25-180</td>
<td>20-170</td>
<td>30-150</td>
<td>30-100</td>
</tr>
<tr>
<td>Vein invasion, no/yes</td>
<td>20/7</td>
<td>94/57</td>
<td>7/4</td>
<td>6/0</td>
</tr>
<tr>
<td>Capsule invasion, no/yes</td>
<td>17/8</td>
<td>104/45</td>
<td>8/2</td>
<td>3/0</td>
</tr>
<tr>
<td>Survival, median months (% 5-year survival)</td>
<td>51 (45.6)</td>
<td>42 (47.0)</td>
<td>- (81.8)</td>
<td>- (100)</td>
</tr>
</tbody>
</table>
Table 3. Multivariate analysis for prognostic factors in M0 ccRCC patients according to the Cox proportional hazard regression model.

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Exp(B)</th>
<th>95% CI of Exp(B)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beginning block</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR^{21/10b} (median high vs median low)</td>
<td>2.599</td>
<td>1.159-5.828</td>
<td>0.02</td>
</tr>
<tr>
<td>Age (&gt;65yrs vs &lt;65yrs)</td>
<td>1.106</td>
<td>0.522-2.343</td>
<td>0.793</td>
</tr>
<tr>
<td>Sex (female vs male)</td>
<td>1.471</td>
<td>0.725-2.985</td>
<td>0.285</td>
</tr>
<tr>
<td>Tumor diameter (&gt;80mm vs &lt;80mm)</td>
<td>2.037</td>
<td>0.991-4.188</td>
<td>0.053</td>
</tr>
<tr>
<td>Tumor nuclear grade (III+IV vs I+II)</td>
<td>2.141</td>
<td>0.848-5.405</td>
<td>0.107</td>
</tr>
<tr>
<td>TNM stage (III+IV vs I+II)</td>
<td>4.048</td>
<td>1.709-9.588</td>
<td>0.001</td>
</tr>
<tr>
<td>After final stepwise analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR^{21/10b} (median high vs median low)</td>
<td>2.624</td>
<td>1.201-5.736</td>
<td>0.016</td>
</tr>
<tr>
<td>Tumor diameter (&gt;80mm vs &lt;80mm)</td>
<td>2.014</td>
<td>1.011-4.013</td>
<td>0.046</td>
</tr>
<tr>
<td>TNM stage (III+IV vs I+II)</td>
<td>4.313</td>
<td>1.963-9.476</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Survivors Deceased

0.01 0.10 1 10 100

miR21/10b versus survivors/deceased

**

M0 M1

Probability of survival according to miR21/10b

p = 0.0095

Probability of survival according to miR21/10b in M0 patients

p < 0.0001

FIGURE 2 Fritz, H.K.M. et al
Supplementary Figure 1. Relative expression of miR in RCC subtypes (n=198) including oncocytoma in comparison with normal kidney tissue (n=50). Levels of miR expression were determined by RT-qPCR and are expressed in arbitrary units as normalized to a set of three internal control short non-coding RNAs. Relative expression in RCC tumors and normal kidney tissue of A, miR-10b; B, miR-21; C, miR-101; and D, miR-223, as determined by RT-qPCR. Kruskal-Wallis test. ****, p < 0.0001; ***, p < 0.001; **, p < 0.01; *, p < 0.05.
Supplementary Figure 2. Pairwise comparison of expression levels in ccRCC tumors and normal kidney tissue of A, miR-10b; B, miR-21; C, miR-101; and D, miR-223, as determined by RT-qPCR. Wilcoxon matched-pairs signed rank test. ****, p < 0.0001; ***, p < 0.001; *, p < 0.05.