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Candidate MicroRNA Biomarkers of Therapeutic Response to Sunitinib in Metastatic Renal Cell Carcinoma: A Validation Study in Patients with Extremely Good and Poor Response

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Abstract. *Background/Aim: Targeted therapy with the tyrosine kinase inhibitor sunitinib is used in the first line of metastatic renal cell carcinoma (mRCC) treatment. The aim of the present study was independent validation of microRNAs (miRNAs) identified in previous studies as biomarkers predicting response to sunitinib therapy. Materials and Methods: Based on a literature search, 10 miRNAs were chosen from six relevant studies as candidates for validation: miR-155, miR-484, miR-221, miR-222, miR-425, miR-133, miR-410, miR-141, miR-628 and miR-942. Validation of these miRNAs was performed on cohort of 56 patients with mRCC with extremely good or poor response responses to sunitinib treatment using quantitative reverse transcription–polymerase chain reaction. Patients were divided into either responding (n=24) or non-responding (n=32) groups to sunitinib treatment according to Response Evaluation Criteria in Solid Tumors and progression-free survival (PFS). All patients in the responding group had PFS longer than 18 months, PFS of non-responders was shorter than 6 months in all cases. Results: miR-942 and miR-133 were confirmed as being differentially expressed in tumors of responding and non-responding patients. It was not possible to validate the predictive value of other tested miRNAs, however, expression of miR-221 and miR-425 tended to be positively associated with therapeutic response (p<0.1). We further developed a model based on the combination of miR-942 and miR-133 expression, that enabled identification of non-responding patients with mRCC with sensitivity of 78% and specificity of 79% (area under the curve=0.8071). Conclusion: Following further independent validation, detection of these miRNAs may prevent unnecessary and costly approaches to therapy in non-responding patients with mRCC.*

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Sunitinib, a tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor receptor (VEGRR) and other members of its receptor family, is currently a first-line treatment in metastatic renal cell carcinoma (mRCC) (1). Despite overall improvement in survival of patients with mRCC treated with sunitinib, there is notable variability in therapeutic response and progression-free survival interval, which is currently not possible to predict (2). Groups of patients with mRCC with primary sunitinib resistance or with resistance developing shortly after treatment initiation are particularly of high clinical interest (3). With accurate predictive biomarkers of response to sunitinib, these patients might be immediately redirected to other therapeutic options such as the mammalian target of rapamycin inhibitor, sorafenib, or new immune checkpoint inhibitors (1).

MicroRNAs (miRNAs) are a group of short (18-25 nt) non-coding RNAs that post-transcriptionally regulate gene expression by complementary binding to 3'untranslated region region of target mRNAs (4). Emerging bioinformatics and experimental evidence suggest their pivotal role in regulation of more than 50% of the human genome (5), and, therefore,

Table I. *Characterization of patients.*

Characteristic	Responders N=24	Non-responders N=32
Gender, n (%)		
Male	19 (79%)	26 (81%)
Female	5 (21%)	6 (19%)
Age, years		
Median (range)	62	63
	40-70	34-83
Histology, n (%)		
Clear cell carcinoma	24 (100%)	32 (100%)
Stage, n (%)		
4	24 (100%)	32 (100%)
PFS on sunitinib therapy		
Median (range) (months)	26,76 (18,36-66,38)	4,51 (2,73-5,72)
Response to sunitinib, n (%)*		
Complete	4 (17%)	0 (0%)
Partial	12 (50%)	0 (0%)
Stable disease	8 (33%)	11 (34%)
Progressive disease	0 (0%)	21 (66%)

PFS: Progression-free survival. *According to Response Evaluation Criteria in Solid Tumors (20).

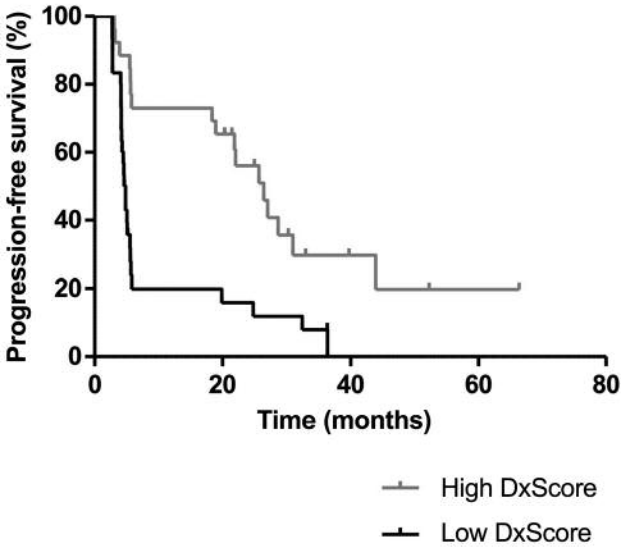

Figure 1. *Survival curve of patients stratified accordingly to miR-133/miR-942-based predictive score (Dx; cut-off value DxScore=−2.467) (p=0.0002).*

involvement of their deregulation in many pathological conditions is not surprising (6, 7). miRNAs are detectable and abundant in a wide range of tumor tissues and body fluids of patients with cancer. As a result, deregulation of tissue or circulating miRNAs might serve as new diagnostic (8), prognostic (9) or predictive biomarkers (10-12) in cancer.

Many studies performed to date indicated strong diagnostic and prognostic potential of miRNA in various cancer types, including RCC (9, 13, 14). However, there are only few reports available focused on the predictive potential of miRNA in mRCC treated with sunitinib (10, 15-19). Although the majority of these studies were based on small cohorts of patients with mRCC, the miRNAs identified partially overlapped. Independent validation of these literature-derived miRNAs as predictive biomarkers in mRCC was, therefore, the main aim of our study. Hopefully, our data serve furthher supporting evidence for miRNAs as potential biomarkers for predicting therapeutic response to sunitinib in mRCC.

Materials and Methods

Study design, patients, and tissue samples. In the present retrospective study, patients with mRCC treated with sunitinib in a standard regimen from four comprehensive cancer centers in the Czech Republic: Masaryk Memorial Cancer Institute Brno, Thomayer Hospital Prague, University Hospital Pilsen and University Hospital Hradec Králové, were included between 2008 and 2014. Local Ethical Committees at all centers approved the study protocol and from written-informed consent was obtained all patients. Hereditary cases of RCC were not included in the study. Response Evaluation Criteria in Solid Tumors (RECIST) (20) and progression-free survival (PFS) interval were used for the definition of the therapeutic response. Out of 224 consecutive patients with mRCC, 56 cases representing the best therapeutic response (PFS longer than 18 months; complete response, partial response or stable disease according to RECIST) and poorest (PFS shorter than 6 months; progressive disease or stable disease according to RECIST) were chosen for a purpose of our validation study (summarized in Table I). Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were collected before treatment administration.

Based on a literature search with the following strategy: "renal cell carcinoma" AND "microRNA" AND ("sunitinib" OR "TKI"), 10 miRNAs were chosen from six relevant studies [10, 15-19] to be evaluated in our study: *miR-155, miR-484, miR-221, miR-222, miR-425, miR-133, miR-410, miR-141, miR-628* and *miR-942*.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR). FFPE samples were deparaffinized and total RNA enriched with short RNA was isolated using commercial mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). RNA concentration and purity were measured using Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). miRNA-specific primers were used in the reverse transcription according to TaqMan miRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed on LightCycler 480 (Roche Life Science, Basel, Switzerland), using TaqMan Universal PCR Master Mix II without uracil-*N*-glycosylase and specific miRNA primer and probe (Thermo Fisher Scientific) for each miRNA. PCR reactions were performed in triplicates, and average threshold cycles and standard deviation values were calculated.

Data normalization and statistical analysis. The expression level of each miRNA in each sample was expressed relative to the average expression of all miRNAs tested. Normalized expression data were

Table II. *miRNA expression levels in tumors of responders and non-responders to sunitinib therapy. Values are relative to the averaged expression of all mRNAs. Significant differences are shown in bold.*

	Median expression (25-75 percentile)				
miRNA	Responders	Non-responders	*p*-Value	Fold change	AUC
miR-155	4.437 (2.20-10.25)	3.246 (1.08-4.92)	0.9594	0.73	0.6579
miR-484	80.98 (30.52-156.00)	49.93 (35.68-128.00)	0.5679	0.62	0.5456
miR-221	2.96 (1.87-5.47)	1.87 (0.88-4.11)	0.0656	0.63	0.6465
miR-222	8.54 (3.54-12.34)	4.54 (2.21-11.91)	0.1069	0.53	0.6284
miR-425	0.62 (0.35-1.13)	0.38 (0.26-0.66)	0.0541	0.61	0.6532
miR-133	**0.11 (0.05-0.30)**	**0.30 (0.17-1.16)**	**0.0474**	**2.73**	**0.6609**
miR-410	0.14 (0.01-0.50)	0.17 (0.05-0.63)	0.3757	1.21	0.5703
miR-141	0.08 (0.02-0.14)	0.06 (0.02-0.15)	0.9934	0.75	0.5013
miR-628	0.09 (0.03-0.12)	0.12 (0.04-0.29)	0.1154	1.33	0.6269
miR-942	**0.07 (0.05-0.14)**	**0.20 (0.15-0.43)**	**0.0003**	**2.86**	**0.7823**

AUC: Area under the curve (receiver operating characteristic analysis).

statistically evaluated by Mann–Whitney *U*-test, ROC analysis and combined Kaplan-Meier analysis (GraphPad Prism 5; GraphPad Software, La Jolla, CA, USA). *p*-Values lower than 0.05 were considered statistically significant.

Results

Expression levels of 10 miRNAs chosen from literature were determined by qRT-PCR in FFPE tumor tissue samples of 56 patients with mRCC treated with sunitinib. Out of 10 miRNAs tested, only *miR-133* and *miR-942* were significantly differently expressed in tumor tissue of patients with different response to sunitinib ($p<0.05$). Both miRNAs were found at higher expression levels in tumor tissue of non-responders, with the *miR-942* level being the most deregulated miRNA of all tested at more than 2.86-fold higher. *miR-221* and *miR-425* tended to show a trend ($p<0.1$) for being down-regulated in tumor tissue of non-responders.

Results of the statistical analysis are summarized in Table II. Logistic regression analysis demonstrated that linear combination of *miR-942* and *miR-133* (DxScore=−1.241-10.2489×*miR-942*-0.2366×*miR-133*; cut-off=−2.467) gave the most reliable predictive model which outperformed other combinations of two or three miRNAs (Figure 1), predicting therapy outcome with an area under the curve (AUC) of 0.8071 with 78.13% sensitivity and 79.17% specificity for identification of non-responders to sunitinib therapy ($p=0.0001$, Figure 2).

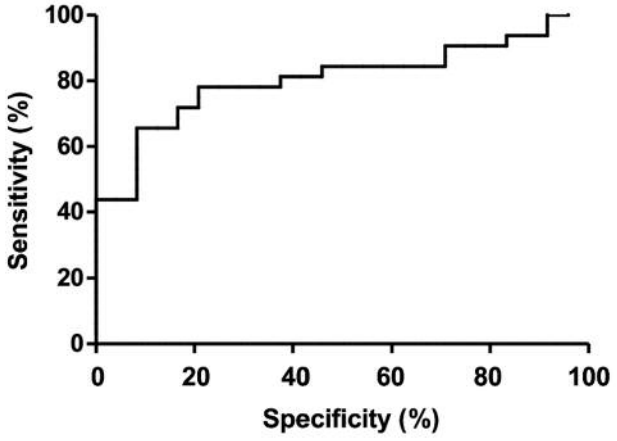

Figure 2. *Receiver operating characteric analysis of the use of the combination of miR-133 and miR-942 in discriminating between responders and non responders to sunitinib treatment (p=0.0001, area under the curve=0.8071).*

Discussion

miRNAs as biomarkers in RCC have been studied with emphasis mostly on their potential diagnostic or prognostic usage. It has been repeatedly shown that selected miRNAs or their combinations can help in sensitive and accurate diagnosis [*e.g. miR-141* (8, 21, 22), *miR-210* (23,24), *miR-21*, *miR-200c* and *miR-429* (4)], and prognostic classification of RCC [*e.g.* studies of Heinzelmann *et al.* (9), Slaby *et al.* (26), Mlcochova *et al.* (27), Fu *et al.* (28)].

There are only a few published studies focused on miRNAs as predictors of therapeutic response to sunitinib in mRCC. Berkers and colleagues found *miR-141* down-regulation and *miR-155* up-regulation in patients with poor response (16). *miR-155* was also by shown to be associated with therapeutic response Khella *et al.* (18) and Merhautova *et al.* (10). In agreement with Merhautova *et al.* (10), Prior *et al.* found *miR-484miR-628-5p, miR-133a* and *miR-942* to be associated with therapeutic response and useful for development of a prognostic model (17). Finally, the most recent and the only sequencing-based study, by García-

Donas *et al*., found *miR-1307-3p* and *miR-425-5p* and their combination to have predictive value in regard to the sunitinib therapeutic response (19). These studies were based on miRNA expression analysis in FFPE tumor tissue specimens. The only study to identify a predictive value for miRNA in blood serum was performed by Gámez-Pozo *et al*. (15)], who identified *miR-410b, miR-1181b* and *miR-424c* to be significantly associated with therapeutic response 15).

In our study, independent validation of the 10 most promising predictive miRNAs identified in the studies published to date was performed. We successfully validated *miR-942* and *miR-133* as being significantly up-regulated in tumors of patients with mRCC with poor response to sunitinib treatment, as shown before in work of Prior *et al*. (17) describing significant association of *miR-484, miR-133, miR-628* and *miR-942* with shorter PFS. We were not able to confirm predictive value of other tested miRNAs, however, *miR-221* and *miR-425* showed a trend for being positively associated with therapeutic response ($p<0.1$). This is in contradiction with results of Khella *et al*. (18) and García-Donas *et al*. (19), who observed miR-221 and miR-425 to be up-regulated in patients with shorter response.

All of the published studies were performed on small cohorts of patients (up to 30 patients) particularly in an exploratory phase, with the exception of García-Donas *et al*.'s study (74 patients in exploratory, 64 in validation phase) (19). This fact might partly explain the low level of overlap between miRNAs identified in the independent studies and the low success rate in the verification of previously identified miRNAs in our cohort.

miR-942 was previously studied, not only in RCC, but also in other types of cancer. In esophageal squamous cell carcinoma, *miR-942* was shown to have oncogenic roles in *in vitro* models and be up-regulated in tumors of patients with poor prognosis (29). According to Liu *et al*., *miR-942* also plays oncogenic roles in hepatocellular carcinoma, where it was inversely correlated with Interferon-stimulated gene 12 (ISG12a) expression (30), which is usually down-regulated in cells resistant to tumor necrosis factor-related apoptosis-inducing ligand induced cell death (29). Regulation of ISG12a by *miR-942* was experimentally shown by Yang *et al*. (31). Only little information is available about the biological roles of *miR-133*, which is known to be involved in muscle development (32) through regulation of extracellular signal-regulated protein kinases 1 and 2 (*ERK1/2*), fibroblast growth factor receptor 1 (*FGFR1*) and catalytic subunit of protein phosphatase 2A (*PP2AC*) genes, as well as cell differentiation and proliferation (33). Information on biological functions of *miR-942* and *miR-133* in relation to the mechanism of action of sunitinib should be the subject of further investigation.

In conclusion, *miR-942* and *miR-133* were found to be a promising predictive biomarkers of therapy response in patients with mRCC. We developed a predictive model based on the combination of these miRNAs which represents a promising tool to be evaluated in independent studies. The functional analysis of *miR-942* and *miR-133* could provide greater insight into their role in therapy resistance or overall disease progression.

Conflicts of Interest

Tomas Buchler and Alexandr Poprach have received honoraria and research support unrelated to this project from Pfizer, Roche, Ipsen, Novartis, BMS and Bayer. The remaining Authors declare no conflict of interest in regard to this study.

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