Thesis

MiR-96-5p level influences cellular growth and survival in colorectal cancer

Submitted by

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Date of birth: 14 March 1988

For the degree of

Doctor medicinae unversiae

(Dr. med. univ.)

At the

Medical University of Graz

Performed at the

Department of Internal Medicine,

Division of Oncology

Supervised by

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Graz, 20rd January 2014 (Anna Lena Ress)

Declaration of Authorship

I declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. Where I have quoted from the work of others, the source is always given at their point of use.
Parts of this work are currently under review in an international peer review journal and will be published in future perspective.

Graz, 20rd January 2014

(Anna Lena Ress)

Acknowledgment

I would like to thank the Medical University of Graz, the German National Foundation and research grants of Dr. Martin Pichler funding this thesis.

I would also like to thank Univ. Prof. Thomas Bauernhofer for his role as a supervisor and his kind words when lab work gets sometimes frustrating. In addition, I have to thank Univ. Prof. Gerald Hoefler for his support of this study in collaboration with Dr. Martin Pichler.

For their excellent technical assistant I offer my gratitude to Verena Stiegelbauer and Elke Winter and all of those who supported me in any respect during the time of work at the University of Chicago and the Division of Oncology, working group Dr. Martin Pichler, of Graz.

Lastly, I would especially like to thank Dr. Martin Pichler, who made this work possible for me, for all his support, motivation, guidance and confidence in my work and my studies over the years.

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Abbreviations

3´UTR	3´untranslated region
5´UTR	5´untranslated region
Akt	Murine thymoma viral oncogene homolog 1
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B
CRC	Colorectal cancer
CSS	Cancer-specific survival
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-mesenchmyl transition
FOXO1	Forkhead box O1
FOXO3	Forkhead box O3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IRS1	Insulin receptor substrate 1
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
MAP4K1	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 1
mCRC	Metastatic CRC
miRNAs	MicroRNAs
mRNA	Messenger RNAs
PFS	Progression-free survival
PRMT5	Methyltransferase 5
pTNM	Pathological tumor-node-metastasis
QRT-PCR	Quantitative Real-Time polymerase chain reaction
RECIST	Response Evaluation Criteria in Solid Tumors
RNU62	RNA U6 small Nuclear 2
UICC	Union for International Cancer Control

Abstract

Background: Expression of miR-96-5p is frequently altered in various types of cancer. However, the biological role of miR-96-5p expression in colorectal cancer (CRC) cells and its ability to predict the clinical course of patients has not been investigated yet.

Methods: We explored miR-96-5p expression in 80 CRC patients and evaluated the impact on clinical outcome. *In vitro* miR-96-5p expression manipulation was performed in CRC cells and the effects on cellular growth, apoptosis and epithelial-mesenchymal transition (EMT)-related gene expression were explored.

Results: Multivariate Cox regression analysis identified low levels of miR-96-5p as independent prognostic factors with respect to cancer-specific survival (hazard ratio=1.8, 95%CI=1.04-3.1, $p<0.035$). *In vitro* overexpression of miR-96-5p led to a reduced cellular growth rate ($p<0.05$), corroborated by a decreased cyclin D1 and increased p27- Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A) expression ($p<0.05$). Forced expression of miR-96-5p in CRC cells entailed no effects on apoptosis or EMT-related genes but decreased the expression levels of the Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) oncogene ($p<0.05$). Despite regulating KRAS expression, there was no significant difference associated with miR-96-5p expression levels and response rates to Epidermal Growth Factor Receptor (EGFR) -targeting agents.

Conclusion: Our data suggest that miR-96-5p influences cellular growth of CRC cells and low expression of miR-96-5p seems to be associated with poor clinical outcome in CRC patients.

Zusammenfassung

Hintergrund: Die sog. miR-96-5p ist eine häufig verändert exprimierte MicroRNA in verschiedenen Krebsarten. Die Rolle beim kolorektalen Karzinom und dem damit zusammenhängenden Krankheitsverlauf des Patienten wurde bis jetzt noch nicht untersucht.
Methoden: Wir haben die Expressionswerte im Gewebe von 80 Patienten mit kolorektalen Karzinom untersucht und den Einfluss auf die klinische Prognose ermittelt. Die miR-96-5p Expression in kolorektalen Zelllinien wurde *in vitro* gezielt beeinflusst und darauf aufbauend die Effekte bezüglich Zellwachstum und Apoptose sowie Gene die bei dem Vorgang der Epithelialen-mesenchymalen Transition (EMT) eine Rolle spielen, untersucht.
Ergebnisse: Die Multivariate Cox Regression Analyse zeigt, dass eine niedrige Expression der miR-96-5p einen unabhängigen prognostischen Faktor in Bezug auf das krebsspezifische Überleben darstellt (Hazard Ratio = 1.8, 95% Konfidenzintervall = 1.04 - 3.1, $p<0.035$).
Eine miR-96-5p Überexpression *in vitro* hat eine verringerte Zellwachstumsrate ($p<0.05$) sowie einer verminderten Cyklin D1 Expression und Anstieg des Cyclin-Dependent Kinase Inhibitor 1A ($p<0.05$) zur Folge. Im Gegensatz zu den gefundenen Effekten auf Zellwachstum hat eine gezielte Reduktion der miR-96-5p keine Effekte auf programmierten Zelltod oder EMT typische Genexpressionmuster, führt jedoch zu einem Abfall der KRAS mRNAExpressionswerten ($p<0.05$). Trotz des Einflusses auf die KRAS Expression haben unterschiedliche miR-96-5p Expressionswerte keine signifikante Assoziation mit dem Ansprechen auf EGFR-Hemmer.
Zusammenfassung: Unsere Ergebnisse zeigen, dass die miR-96-5p das Zellwachstum von kolorektalen Krebszelllinien beeinflusst und eine verringerte Expression im Krebsgewebe im Zusammenhang mit schlechterer Prognose bei Patienten mit kolorektalen Karzinom steht.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and ranks second in females, with 143,460 new cancer cases and 51,690 deaths estimated to occur in 2012 in the United States [1]. Recently, the introduction of novel treatment options, including the monoclonal antibodies cetuximab and panitumumab which are directed against the epidermal growth factor receptor (EGFR), have improved the survival time of patients with metastatic CRC (mCRC) [2-4]. Despite the development of these relatively expensive novel agents, early reports demonstrated that only 10 to 20% of unselected patients with mCRC clinically benefited from EGFR-targeting monoclonal antibodies [2]. Subsequent analysis of large clinical trial data demonstrated a clear negative relationship between objective response rates and the occurrence of mutations in the Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS)* gene, which led to the establishment of *KRAS* mutations as a negative predictor for EGFR-targeted therapies in mCRC [5-7]. However, the occurrence of *KRAS* mutations only accounts for approximately 35 to 45% of non-responsive patients [8], therefore other potential biomarkers are required to identify non-responsive patients. Given the toxic side effects and the high costs of these agents, a clear need for further evaluation of potential prognostic and predictive factors remains [8]. Recently, the classical dogma stating that protein-coding genes recognized as tumor suppressors and oncogenes are key factors implicated in carcinogenesis was expanded by the identification of a class of non-protein-coding RNA molecules known as microRNAs (miRNAs) [9]. Their general mechanism of action involves the endogenous suppression of gene expression through the binding of the 3´ untranslated region (3´UTR), the coding region or the 5´ untranslated region (5´UTR) of larger target messenger RNAs (mRNA) as well as impairment of protein translation [9]. Expression levels of several miRNAs are altered in CRC [10-12]. One of these, miR-96-5p, belongs to the miR-183-96-182 gene cluster and was identified as differently expressed in many human cancers including lymphoma [13], hepatocellular carcinoma [14], breast cancer [15], prostate cancer [16] and urothelial carcinoma of the bladder [17]. Interestingly, it has been shown that miR-96-5p directly targets the mRNA of the *KRAS* oncogene and functions as

a tumor-suppressing miRNA in pancreatic cancer cells [18]. Ectopic expression of miR-96-5p through a synthetic miRNA precursor inhibited KRAS protein expression, dampened v-akt murine thymoma viral oncogene homolog 1 (Akt) signaling and triggered apoptosis in pancreatic cells [18]. Recently, Loboda et al. demonstrated that activation of the RAS signaling pathway, assessed by measuring gene expression levels of members of this signaling pathway, can predict resistance to cetuximab therapy in CRC [19]. As miR-96-5p was shown to target KRAS mRNA and therefore influences the RAS signaling pathway in CRC cells, the regulation of KRAS by miR-96-5p might contribute to tumor progression and resistance against EGFR-targeting agents. In this context, a previously published study showed that a specific miRNA, miR-212, also regulates resistance against cetuximab in head and neck squamous cell carcinoma [20]. In addition, data from Garofalo et al. indicated that particular miRNAs regulate resistance against EGFR inhibitors in lung cancer [21]. Given the previously reported altered expression of miR-96-5p in various types of human cancer and the effect on KRAS expression, evaluation of its potential influence on prognosis and its possible role in predicting therapeutic response in CRC patients treated with EGFR-targeted agents is of great interest. In this study, we compared the miR-96 expression in CRC tissue to normal colon tissue. Based on the expression differences, we analyzed whether alterations in miR-96 expression influence the prognosis of CRC patients. To get more insight into the biological mechanisms we performed *in vitro* miR-96-5p manipulation and evaluated effects on proliferation, apoptosis and gene expression. Finally, we selected a sample collection of patients who were treated with EGFR-targeting agents and evaluated the ability of miR-96-5p to predict the response to this therapeutics.

Material and Methods

Study population

Our retrospective study included data from 80 histologically confirmed CRC patients who underwent treatment at the Division of Oncology, Medical University of Graz between January 2005 and January 2012. The ethics committee of the Medical University of Graz has approved this study (No. 23-473 ex 10/11). Patients' clinico-pathological data were retrieved from medical records at the same institution. All cases were reviewed based on pathology reports and histological slides for pathological tumor-node-metastasis (pTNM) categories. The medical records were particularly searched for the presence or absence of distant metastases (M stage), the Union for International Cancer Control (UICC) tumor stage (I-IV) and the number and characteristics of treatment lines.

To test the influence of miR-96-5p expression on the objective response to EGFR-targeted therapy, patients were selected based on evidence that treatment outcome could be attributable only to administration of either panitumumab or cetuximab. This included 54 patients who received either cetuximab or panitumumab as monotherapy and patients who experienced disease progression while on previously used chemotherapy, where cetuximab or panitumumab were added to the initial chemotherapy. In all patients, refractoriness to prior chemotherapy was defined as documented disease progression after comparison of computed tomography or magnetic resonance imaging scans after 4 cycles (administered for at least 8 weeks) of chemotherapy. The disease state was evaluated at baseline and after every four cycles of therapy (8 weeks) during treatment. Therapy response was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) [22].

KRAS mutational testing

To test samples for mutations in exon 2 of *KRAS*, we extracted DNA from tumor samples and determined the sequence in codons 12 and 13 by pyrosequencing. It should be noted that we used pyrosequencing because its high sensitivity results in a more accurate assessment of the *KRAS* mutation burden in CRC [23]. Briefly, histological sections of each case were reviewed by pathologists Stephan Jahn, Sigurd Lax, Gerald Höfler and one paraffin block of each tumor tissue and corresponding non-neoplastic colon mucosa was retrieved. Genomic DNA was extracted as previously described [24]. The quality and concentration of the extracted DNA was determined using a NanoDrop instrument (Thermo Scientific, Wilmington, DE). Pyrosequencing analysis of the samples was performed according to the manufacturer's recommendations for the PyroMark Q24 KRAS assay (Qiagen, Hilden, Germany) on a PyroMark Q24 instrument with PyroMark Q24 1.0.9 software (Qiagen, Hilden, Germany Qiagen).

Colorectal cancer cell lines

For confirmation of miR-96-5p expression in CRC cells, we used two human CRC cell lines (Caco-2 and HRT-18). The Caco-2 cell line expresses the wild-type *KRAS*, and in the HRT-18 line, *KRAS* is mutated. Cell lines were grown under standard conditions [24] and after harvesting they were fixed in formalin and embedded in paraffin. MiRNAs were extracted by the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) as described below. The expression of miR-96-5p was normalized using RNU6B. Relative expression was compared to a pool of normal colon mucosa [25].

Quantification of miR-96-5p expression level

RNA was extracted from both tumor tissue and non-neoplastic colon mucosa (usually from the resection margins). Two to eight 10 μm-thick tissue sections were used for manual micro dissection to obtain areas with at least 60% tumor cells. MiRNAs were isolated using the miRNeasy FFPE Kit 50 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. CDNA was synthesized from 500ng of RNA using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantification of miRNAs was performed using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) and the Hs_ miR-96 miScript Primer

Assay (Qiagen, Hilden, Germany) according to the manufacturer's recommendations on a Light Cycler 480 real-time PCR device (Roche, Mannheim, Germany). A quantitative Real-Time polymerase chain reaction (QRT-PCR) was carried out using normalization to RNA U6 small Nuclear 2 (RNU62) as recently published [26]. Differences in fold expression with regard to the adjacent normal colon mucosa were calculated from the duplicate of *C*T values following the $2^{-\Delta\Delta CT}$ method [25]. For detection of miR-96-5p from cell lines after transfection experiments, 1µg of total RNA was reverse transcribed by the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. The Hs_miR-96_1 miScript Primer Assay (Mature miRNA sequence: 5' UUUGGCACUAGCACAUUUUUGCU, Qiagen, Hilden, Germany) and for normalization the Hs_RNU6-2_1 miScript Primer Assay (Qiagen, Hilden, Germany) were applied on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics) using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany).

In vitro transfection experiment of miR-96-5p mimetics/inhibitors

The human colorectal carcinoma cell line HCT116 was commercially purchased from American Type Culture Collection. Cells were grown in McCoy's 5A modified Medium (w/o L-Glutamine, 2,2g/L sodium bicarbonate, from Biochrome) containing 10% fetal bovine serum gold (PAA) and antibiotics (penicillin and streptomycin) and were incubated in a 5% CO_2 humidified incubator at 37°C. For a transient transfection approach with the aim to reduce or overexpress the miR 96-5p expression, HCT116 cells were transfected using the fast forward transfection protocol as suggested by the HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) protocol according to the manufacturer´s instructions. A specific miR-96-5p inhibitor (Anti-Hsa-mir96-5p, Qiagen, Hilden, Germany) and mimetic (Syn-Hsa-mir96-5p, Qiagen, Hilden, Germany) were commercially purchased. For the reference to normalize the findings we used the miScript negative Control (Qiagen, Hilden, Germany) under the same concentrations and conditions as used for the inhibitor/mimetic [10-50nM]. For confirmation of transfection efficacy, the AllStars cell death control (Qiagen, Hilden, Germany) was used. For measuring gene expression after miR-96-5p inhibition/overexpression, we seeded $2x10^5$ HCT116 cells in complete medium in 6-well plates. MiRNA inhibitor/mimetic-transfection complexes were added according to the fast forward transfection protocol as

recommended by the manufacturer (HighPerfect Transfection Reagent, Qiagen, Hilden, Germany). Transfected cells were incubated under their normal growth conditions (37°C, 5% CO_2) and the effect of miR-96-5p manipulations on changes in gene expression levels was measured by qRT-PCR after 48 hours. Gene expression was evaluated with regard to proliferation-related genes (cyclin D1 and p27-CDKN1B), EMT-related genes (e-cadherin and vimentin) as well as the KRAS oncogene. For mRNA quantification, up to 1µg of total RNA was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Quantitative RT-PCR was carried out in triplicates for each sample using commercially available primers (QuantiTect Primer Assay, Qiagen, Hilden, Germany) specific for the proliferation markers cyclin D1 (Hs_CCND1_1_SG) and cyclin-Dependent Kinase Inhibitor 1B (CDKN1B) (Hs_CDKN1B_2_SG), EMT-related genes (E-cadherin, Hs_CDH1_1, Vimentin, Hs_VIM_1_SG) and KRAS (Hs_KRAS_1_SG) and the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Hs_GAPDH_1_SG) on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics) using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). GAPDH was used for normalization and relative gene expression levels were calculated with the delta delta CT method. For each condition, cells were seeded in six-well plates and each experiment was independently repeated three times.

WST-1 proliferation assay

To test whether altered miR-96-5p expression influence cellular growth rates of CRC cells, we performed overexpression experiments with a miR-96-5p mimetic (Syn-Hsa-mir96-5p, Qiagen, Hilden, Germany) and measured the cellular growth by applying the WST-1 proliferation assay. In more detail, after standard trypsinisation $2x10^4$ HCT116 cells per well were seeded in a 96-well culture plate. After transfection with a miR-96-5p mimetic, cells were incubated in normal McCoy`s 5A modified Medium growth medium (Biochrom, Berlin Germany) for 48 hrs and the WST-1 proliferation reagent (Roche Applied Science, Mannheim Germany) was applied according to the manufacturer’s recommendations. After 4 hours the colorimetric changes were measured using a SpectraMax Plus (Molecular Devices) at a wavelength of 450 nm with a reference wavelength at 620 nm. Three independent experiments were performed.

Apoptosis assay

To clarify the role of miR-96-5p in apoptosis, we carried out a commercially available apoptosis assay after overexpression of miR-96-5p. In more detail, $2x10^4$ HCT116 cells were seeded in each well of a 96 well plate and were transiently transfected with Syn-Hsa-mir96-5p (Qiagen, Hilden, Germany) and Anti-Hsa-mir96-5p (Qiagen, Hilden, Germany). Caspase activity was measured after 24 and 48 hours in HCT116 cells using the Caspase-Glo 3/7 Assay Systems (Promega, Mannheim Germany) according to manufacturer's instructions. Luminescence was measured in a POLARstar OPTIMA microplate reader (BMG Labtech). Three independent experiments were performed.

Statistical analyses

All statistical analyses were performed using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA) and made possible through the help of Martin Pichler. Fisher's exact test, Chi-squared test, Mann-Whitney and Student's t-test were used to analyze the association between miR-96-5p expression and clinico-pathological parameters. Cancer-specific survival (CSS) was defined as the time from date of diagnosis to the date of cancer-related death. CSS was assessed using the Kaplan-Meier method, and the log-rank test was performed to compare the survival curves of individual groups. Univariate and multivariate Cox proportional hazards models including age, gender, tumor stage (according to the AJCC/UICC 2010 TNM classification), tumor grade, level of CEA, number of therapies and miR-96-5p expression were used to determine the clinico-pathological parameters that were statistically significant for CSS. The reported results included hazard ratios (HR) and 95% confidence intervals (CI). The objective tumor response rate after 8 weeks of treatment with an EGFR-targeting agent was the end point regarding the predictive value of miR-96-5p expression. Qualitative comparisons of the objective response to therapy after 8 weeks of treatment and miR-96-5p expression as a predictor were performed by the two-tailed Fisher's exact test to check possible significance. A p-value of <0.05 was considered as statistically significant. Progression-free survival (PFS) was calculated from the date therapy with an EGFR-targeting agent began to the most recent available documentation of disease progression according to the RECIST criteria.

Results

Clinico-pathological parameters of the study cohort are summarized in Table 1. Mean age was 60 years (SD ± 10.1, range: 31–77). Median follow-up was 39 months (interquartile range: 25–61). Of the 80 patients, 66 (82.5%) died due to their underlying malignant disease during the follow-up period.

Table 1. Clinico-pathologic characteristics of the colorectal cancer patients (n=80) included in this study.

Clinico-pathologic parameters	Patients (n=80)	Proportion
Gender		
Male	54	67.5%
Female	26	32.5%
Age at diagnosis		
≤60	37	46.3%
>60	43	53.7%
Tumor location		
colon	50	62.5%
rectum	30	37.5%
Tumor grade		
G1	2	2.5%
G2	58	72.5%
G3	20	25.0%
Stage at diagnosis		
II-III	35	43.8%
IV	45	56.3%
Lines of therapy		
<3	25	31.3%
≥3	55	68.7%

In both HRT-18 and Caco2 CRC cell lines, miR-96-5p was significantly up regulated when compared to normal colon mucosa (p<0.001, data not shown). This result prompted us to measure miR-96-5p expression levels in the whole cohort of 80 CRC patients. The mean expression level of miR-96-5p was 5.23-fold (ranging from 0.44 to 19.22) higher in neoplastic tissue compared to normal non-neoplastic colon mucosa (p<0.001). The median neoplastic:non-neoplastic miR-96-5p expression level (which was 3.64 up-regulation compared to normal colon mucosa) was used to classify patients as low miR-96-5p-expressing (below the 50th percentile) and high miR-96-5p-expressing (above the 50th percentile). The median is widely used as a cut off value to study clinical effects of miRNA expression data [26] and was used throughout the study. In the study, miR-96-5p expression levels did not correlate significantly with patient age, gender, stage, tumor grade or the level of tumor markers (all p-values >0.05, Table 2), but a low miR-96-5p expression was significantly associated with distant metastasis at the time of diagnosis (p=0.025, Table 2).

Table 2. Correlations between miR-96 expression and clinico-pathological parameters.

Clinico-pathological parameters		MiRNA-96 expression		
		MiRNA-96 low (n=40)	MiRNA-96 high (n=40)	p-value
Age	≤60 >60	16 (40%) 24 (60%)	21 (52.5%) 19 (47.5%)	0.262
Gender	male female	28 (70%) 12 (30%)	26 (65%) 14 (35%)	0.633
Tumor Location	colon rectum	23 (57.5%) 17 (42.5%)	27 (67.5%) 13 (32.5%)	0.356
Tumor Grade	G1 G2 G3	1 (2.5%) 26 (65%) 13 (32.5%)	1 (2.5%) 32 (80%) 7 (17.5%)	0.298
Stage	II-III IV	14 (35%) 26 (65%)	21 (52.5%) 19 (47.5%)	0.115
Distant metastasis at diagnosis	No Yes	13 (32.5%) 27 (67.5%)	23 (57.5%) 17 (42.5%)	0.025
Levels of tumor marker (Median ng/ml)	CEA	15.6	4.1	0.544
Lines of therapy	<3 ≥3	16 (40%) 24 (60%)	9 (22.5%) 31 (77.5%)	0.091

Legend
CEA: carcinoembryonic antigen

Univariate analysis identified high tumor grade (G3 versus G1+G2), advanced tumor stage (stage IV versus stage II-III) and low expression of miR-96-5p as poor prognosticators for CSS (all *p*-values <0.05, Table 3), whereas age, gender, number of therapy lines and location of the tumor were not significantly associated with CSS (Table 3).

Table 3. Univariate and multivariate analyses of clinico-pathological parameters for the prediction of cancer-specific survival in patients with colorectal cancer.

	Cancer-specific survival			
	Univariate		Multivariate	
Parameter	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age at diagnosis (yrs.) ≤60 >60	1 (reference) 1.27 (0.77-2.10)	0.354	1 (reference) 1.10 (0.65-1.83)	0.732
Gender Male Female	1 (reference) 1.06 (0.62-1.80)	0.832	1 (reference) 1.19 (0.68-2.10)	0.526
Tumor Location Colon Rectum	1 (reference) 0.80 (0.49-1.32)	0.389	n.d.	
Tumor grade G1+G2 G3	1 (reference) 2.60 (1.48-4.58)	<0.001	1 (reference) 2.10 (1.14-3.77)	0.017
Tumor Stage II+III IV	1 (reference) 2.10 (1.24-3.47)	0.006	1 (reference) 1.93 (1.11-3.38)	0.020
Lines of therapy ≤3 >3	1 (reference) 0.60 (0.35-1.07)	0.053	n.d.	
MiR-96 Expression high low	1 (reference) 2.20 (1.32-3.65)	0.002	1 (reference) 1.80 (1.04-3.10)	0.035

Legend
n.d. not done because of non-significance in univariate analysis

The median CSS for patients with high miR-96-5p expression was 50.5 months, whereas the median CSS for patients with low miR-96-5p expression was 32.5 months (*p*=0.013). Figure 1 shows the Kaplan-Meier curve for CSS and reveals that low miR-96-5p expression is a factor for poor prognosis in CRC (*p*=0.002, log-rank test).

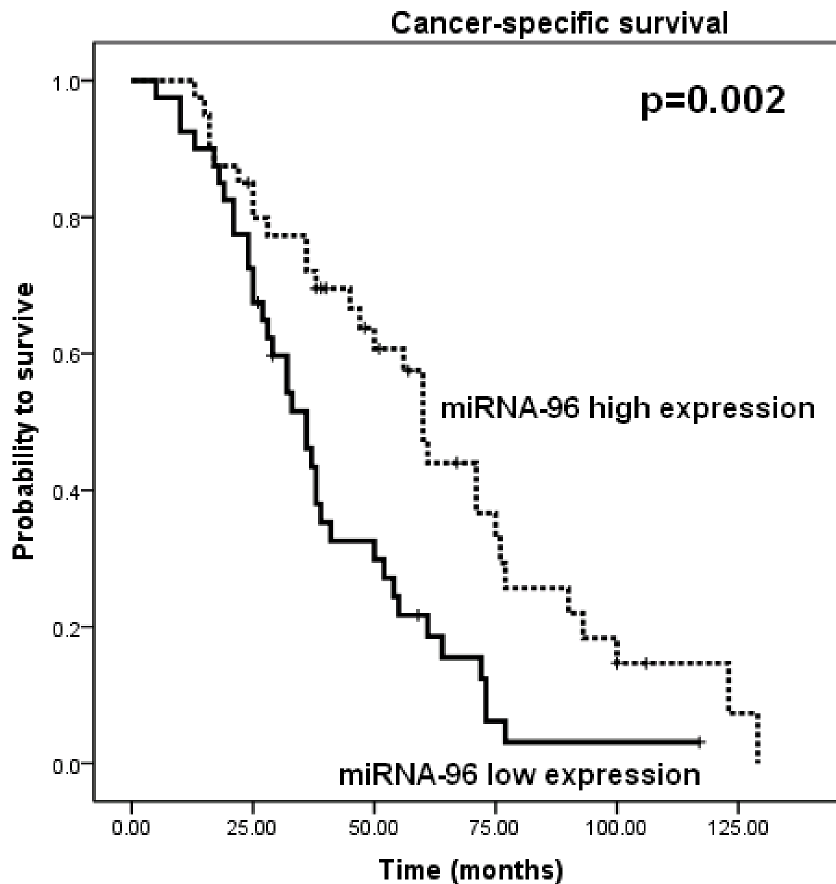

Figure 1. Kaplan-Meier plot for cancer-specific survival in colorectal cancer patients (n=80) stratified by miR-96-5p expression level.

To test whether the prognostic value of low miR-96-5p expression was independent of other risk factors for poor survival, a multivariate analysis was performed using a Cox proportional hazard model. Multivariate analyses including age, gender and all factors significant in univariate analysis (tumor stage, tumor grade and level of miR-96-5p expression) demonstrated that low miR-96-5p

expression was an independent predictor for poor CSS in CRC patients (HR=1.8, CI=1.04-3.1, *p*<0.035, Table 3).

In an attempt to further clarify the association of impaired clinical outcome in CRC patients with low miR-96-5p expression, we conducted *in vitro* overexpression/inhibition experiments with miR-96-5p mimetic/inhibitor. A WST-1 proliferation assay was used to investigate the effect of miR-96-5p manipulation on cellular growth of the transfected HCT-116 cells. After 48 hours, we observed a statistically significant decrease in the growth of miR-96-5p overexpressing cells in comparison to cells treated by the reference or the miR-96-5p inhibitor (25 ± 5% decrease, *p*<0.05, Figure 2).

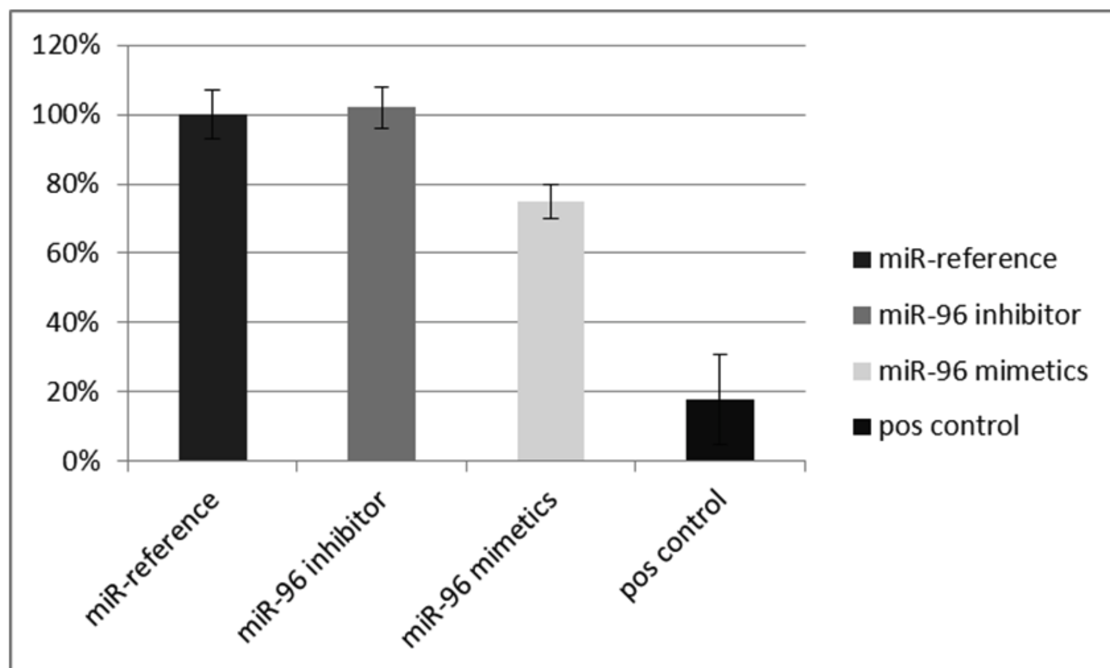

Figure 2. MiR-96-5p overexpression leads to a decrease of cellular growth rate measured by WST-1 assay (25% ± 5% reduction, *p*<0.05) compared to reference control and miR-96-5p inhibitor.

Consecutively, we measured the proliferation markers cyclin D1 and p27 gene expression in miR-96-5p overexpressing cells. We detected a significantly decreased expression of the proliferation marker cyclin D1 (34 ± 3% decrease, $p<0.05$, Figure 3 A) and a significant increase of the cell cycle inhibitor p27 (1.6 fold increase, $p<0.05$, Figure 3 B).

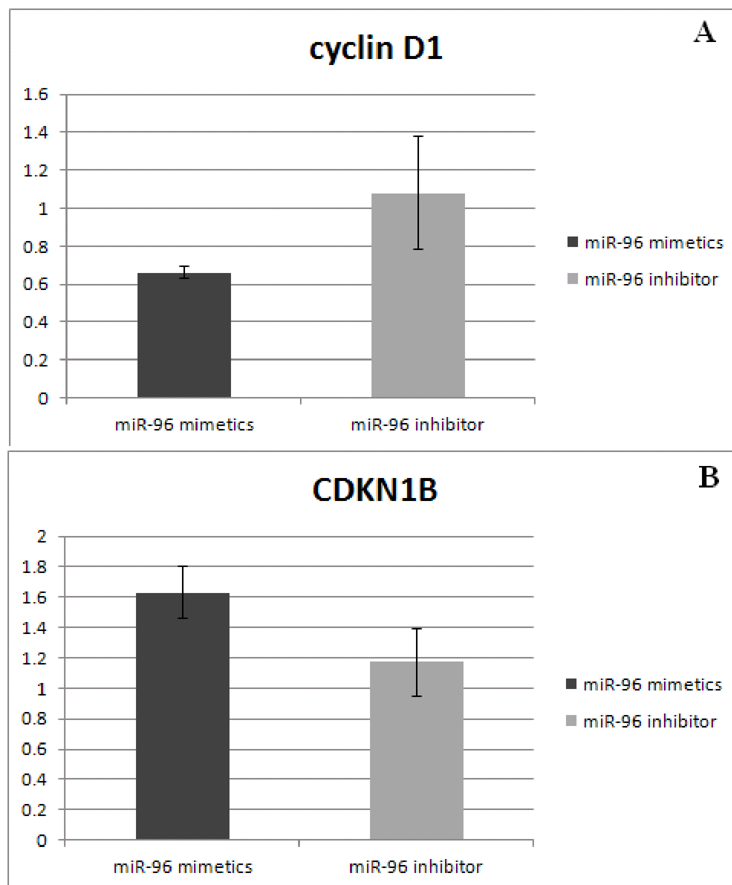

Figure 3. MiR-96-5p overexpression leads to a significant decrease of the proliferation marker cyclin D1 (A) and a significant increase of the cell cycle inhibitor p27-CDKN1B (B) ($p<0.05$).

Regarding apoptosis activity and the expression of the EMT-markers e-cadherin and vimentin, we could not observe any differences in miR-96-5p overexpressing HCT-116 cells compared to inhibited cells (data not shown).
KRAS oncogene expression was significantly reduced after miR-96-5p overexpression (33 ± 6% decrease, $p<0.05$, Figure 4).

Figure 4. MiR-96-5p overexpression significantly ($p<0.05$) reduces KRAS oncogene expression levels.

To analyze whether the KRAS-targeting miR-96-5p expression has an impact on the efficacy of EGFR-targeting therapy and may therefore predict the therapeutic response to these drugs, we analyzed patients whose treatment outcome could be attributable only to administration of either panitumumab or cetuximab. Fifty-four patients were analyzed using the criteria mentioned in detail in the methods section. Overall, 24 patients were treated with a cetuximab-containing therapy and 30 patients were treated with a panitumumab-containing therapy. Eighteen patients received monotherapy with either panitumumab (n=16) or with cetuximab (n=2), whereas 36 patients, who were refractory to the previous chemotherapeutic regime, received the monoclonal antibodies in combination with prior chemotherapy. Overall, there were 26/54 (48.1%) patients with progressive disease, 17/54 (31.5%) with stable disease, 10/54 (18.5%) with partial remission and 1/54 (1.9%) with complete remission according to the internationally established RECIST criteria after receiving 4 cycles of an EGFR-targeted

containing regime. The mean PFS time was 4.24 months (ranging from 2 to 18 months). There were no significant differences found regarding miR-96-5p expression and therapeutic response with an EGFR-targeting agent (*p*=0.64, Table 4).

Table 4. Therapy response in CRC patients treated by an EGFR-targeted containing treatment regime.

	MiRNA-96 expression	
RECIST criteria	**Low expression**	**High expression**
Complete remission	1 (3.7%)	0 (0.0%)
Partial remission	5 (18.5%)	5 (18.5%)
Stable disease	7 (25.9%)	10 (37.0%)
Progressive disease	14 (51.9%)	12 (44.4%)

The median PFS in the group of high miR-96-expressing patients was 3 months compared to median PFS of 2 months in the group of low miR-96-expressing patients. However, this difference was not statistically significant (*p*=0.320, log-rank test, data not shown).

Discussion

CRC represents one of the most common types of cancer that often leads to impairment of quality of life and subsequent death. Therefore, novel biomarkers that might predict the clinical outcome of CRC patients would provide a valuable tool for proper risk stratification and patient surveillance and ideally provide a basis for decision making to avoid a potentially harmful, expensive and ineffective therapy. In this study, we first confirmed the expression and up-regulation of miR-96-5p in CRC using different CRC cell lines and then compared the difference in miR-96-5p expression in CRC tissue and its corresponding non-neoplastic mucosa. Overall, a significant up-regulation of miR-96-5p (mean 5.23 fold) was observed in CRC tumor samples compared to paired samples of non-tumor tissue. These data are in line with a previously published study that reported an average of 3.64 fold up-regulation of miR-96 in 15 CRC cell lines compared to a non-neoplastic cell line [10]. In addition, Sarver et al. performed an array-based quantification of differentially expressed miRNAs in CRC tissue and found a 2.04 fold up-regulation of miR-96 in CRC tissue [27]. Most recently, Xu and colleges confirmed an up-regulation of miR-96 and an association with liver metastases on 52 CRC patients [28]. These findings of significant up-regulation of miR-96 in CRC tissue should encourage further studies that address the important question of whether miR-96 might be useful as a diagnostic marker in body fluids such as blood or stool for early detection of CRC. This concept has been successfully tested for miR-96 in urothelial cancer of the bladder, in which the up-regulation in cancer tissue is correlated with high stage and grade and might serve as a diagnostic marker in the urine [17,29]. To explore a possible pathophysiological mechanism of the differential expression of miR-96-5p in CRC, we measured cellular functions including cell growth, apoptosis and gene expression markers of proliferation and EMT after *in vitro* overexpression/inhibition experiments. The miR-96-5p overexpressing cells showed a significant decrease of cellular growth. In line with this data, expression of the proliferation marker cyclin D1 was reduced and expression of the cell cycle inhibitor p27 was significantly increased. These *in vivo* and *in vitro* data support the hypothesis that miR-96-5p might act as a tumor suppressive miRNA in CRC. A given miRNA may function as a tumor suppressor

or oncogene (“onco-miR”). Which function it adopts, depends on the molecular and cellular context. For miR-96-5p, both a role as a tumor suppressor and an onco-miR has been proposed. For instance, miR-96 targets the tumor suppressor gene forkhead box O3 (FOXO3) and thereby mediates protection against apoptosis and promotes cell survival in breast cancer cells [30]. Additionally, overexpressed miR-96 reduces the expression of the tumor suppressor forkhead box O1 (FOXO1) in endometrial cancer and thus induces deregulated cell cycle control and impaired apoptotic responses, which may be central to endometrial tumorigenesis [31]. In bladder cancer, overexpression of miR-96 may affect growth of bladder cancer cells by up-regulating insulin receptor substrate 1 (IRS1) and Mitogen-Activated Protein Kinase Kinase Kinase Kinase 1 (MAP4K1) levels [29]. Moreover, a recently published case control study including 70 paired samples from lung cancer patients demonstrated that high expression levels of miR-96 in tumor tissue and serum are associated with poor overall survival in lung cancer patients. In contrast to these studies, which support the role of miR-96 as an onco-miR, some reports proposed an opposite role for miR-96 in other cancer types. Pal et al. showed that the chromatin modifying factor protein arginine methyltransferase 5 (PRMT5) is elevated in mantle cell lymphoma and that down-regulation of miR-96 in this lymphoma contributes to altered expression of PRMT5 [13]. In another study regarding pancreatic cancer, Yu and colleagues demonstrated that miR-96 acts as a tumor suppressor [18]. In this study, the authors established that miR-96 decreases cancer cell invasion and migration and slows tumor growth in vivo [18]. Interestingly they described a KRAS-inhibiting effect of miR-96 and concluded that miR-96 might provide a novel therapeutic strategy for KRAS-driven cancers [18]. To date, no study has described the functional role or prognostic value of miR-96-5p regarding CRC patients. The prominent role of miR-96-5p in different types of cancer and the KRAS-interacting properties of miR-96-5p in pancreatic cancer prompted us to investigate the potential role of miR-96-5p as a biomarker to predict the response of EGFR-targeting agents in *KRAS* wild-type CRC patients. After confirming that miR-96-5p overexpression leads to a reduced *KRAS* oncogene expression we measured miR-96-5p in 54 paired samples of CRC patients but could not identify an association of miR-96-5p expression and response to EGFR-targeting agents in our cohort.

In conclusion, this study indicates that miR-96-5p expression influences cellular growth rate of CRC cells and low expression of miR-96-5p in colorectal cancer is an independent negative prognostic factor for colorectal cancer patients. Further pre-clinical studies are warranted for the validation of miR-96 as a novel diagnostic, prognostic and therapeutic biomarker in CRC patients.

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