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Prognostic potential of circulating miR-93-5p in patients with colorectal cancer liver metastases

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This study aimed to examine the expression pattern of tumoral and circulating miR-93-5p in patients with colorectal cancer (CRC) liver metastasis (CRLM) and to explore its predictive and prognostic potential. CRLM tissue, surrounding non-tumor liver tissue, and serum were obtained from 35 patients with CRLM. The expression pattern of tissue and circulating miR-93-5p in patients with CRLM was determined using quantitative polymerase chain reaction, using miR-16-5p for normalization. Sample-based cut-off values for CRLM and serum miR-93-5p expression were calculated using Receiver Operating Characteristic curve analysis to stratify the patients into high and low miR-93-5p expression groups which were that compared with patients' clinicopathological data, therapy response, one-year disease-free survival, and disease recurrence. Relative miR-93-5p expression was higher in CRLM in comparison to the non-metastatic liver tissue ($p<0.001$). CRLM miR-93-5p expression showed moderate negative correlation with carcinoembryonic antigen levels ($r=-0.406$; $p=0.016$). There were no differences in high-/low-miR-93-5p expression and therapy responders vs. non-responders, which was confirmed *in vitro* using metastatic and normal colonic cells SW620 and HCEC-1CT, respectively. No difference was observed in one-year recurrence-free survival in patients with high vs. low miR-93-5p expression in CRLM or serum. However, high miR-93-5p serum levels were significantly associated with early disease recurrence ($p=0.035$). In conclusion, miR-93-5p serum levels could be potentially used as a prognostic factor for early disease recurrence in CRLM patients.

Key words: metastatic colorectal cancer, colorectal cancer liver metastasis, microRNA, miR-93-5p, prognostic biomarker

Colorectal cancer (CRC) takes third and second place in terms of incidence and mortality rates worldwide, respectively [1]. Approximately one-quarter of CRC patients are metastatic at the time of diagnosis (metastatic CRC, mCRC) [2]. Almost half of CRC patients will develop metastatic tumors eventually [3], with the liver being the most common target organ [2, 4]. The presence and progression of colorectal cancer liver metastasis (CRLM) can be a life-limiting event since it accounts for approximately half of deaths in CRC patients, thus contributing to high CRC mortality rates [5]. Hepatic resection of colorectal cancer liver metastases is crucial for treatment and leads to a 5-year overall survival rate of up to 58% [6–8]. However, the majority (75–90%) of metastatic CRC patients are not candidates for resection because of disease extent or severe comorbidities [6, 8]. For this group of patients, systemic neoadjuvant chemotherapy is administrated with an aim to render unresectable disease resectable.

Systemic chemotherapy has long been the gold standard of chemotherapy for mCRC patients. Most commonly, 5-fluorouracil (5-FU) is combined with folinic acid and oxaliplatin (FOLFOX regime) [9], or with folinic acid and irinotecan (FOLFIRI regime) [10]. However, since 2004 the decision-making process regarding the choice of first-line therapy for mCRC has been complicated by the introduction of molecularly targeted biological agents: bevacizumab (the monoclonal antibody (mAb) against vascular endothelial growth factor (VEGF)) and cetuximab and panitumumab (mAbs targeting epidermal growth factor receptor (EGFR)) [11]. Although mentioned biologicals improved survival rates in patients with mCRC and the overall survival is now in the 30-month range [12], cetuximab and panitumumab are only beneficial in *KRAS*/*NRAS* wild-type patients [13, 14], while for bevacizumab there are no validated predictive biomarkers available [3]. As, for prognostic biomarkers,

it has been shown that *BRAF* mutant mCRC tumors had a poorer prognosis in comparison to *BRAF* wild-type tumors [15]. Albeit the new biomarkers are rapidly emerging, the negative predictive role of RAS status for anti-EGFR therapy and the negative prognostic role of *BRAF* mutations are currently the only ones with a clear clinical utility [16, 17]. For this reason, it is imperative to find new predictive and prognostic biomarkers in order to personalize the treatment of patients with mCRC.

microRNAs (miRNAs) are small regulatory RNA molecules that direct posttranscriptional repression of mRNA targets [18]. Expression of miRNAs is altered in CRC in comparison to normal mucosa [19]. Furthermore, microRNA signatures are associated with the diagnosis, staging, progression, and prognosis of CRC [20]. Additionally, miRNAs expression has been associated with therapeutic response in CRC [21]. Having that previously in mind, and a fact that the miRNAs have high tissue specificity, stability, and altered expression in tumor development, miRNAs have been suggested as diagnostic, prognostic, and predictive biomarkers in CRC, both in tissue and as non-invasive circulating molecules in the blood [21–23].

miR-93-5p belongs to the miR-106b-25 cluster located at chromosome 7q22, in the intron 13 of the *MCM7* gene [24]. There is no consensus regarding the direction of miR-93-5p expression in CRC in comparison to normal mucosa, with some studies showing that there is a downregulation of miR-93-5p [25, 26], while others show upregulated miR-93-5p expression [27]. Additionally, there are no consistent reports of whether miR-93-5p is an oncogenic [28] or a tumor-suppressor miRNA [29]. In our previous study, we have shown that expression of miR-93-5p was downregulated in response to chemotherapy for mCRC in SW620 cells [30]. Two studies have shown that decreased tumoral expression of miR-93 could be used as a novel prognostic factor for CRC [25, 31]. However, the predictive or prognostic role of miR-93-5p in mCRC has not yet been elucidated. This study aimed to examine the expression pattern and predictive and prognostic potential of tumoral and circulating miR-93-5p in patients with CRLM.

Patients and methods

Subjects. In this study, thirty-five patients with CRLM were recruited between December 2016 and April 2019. All subjects were treated by curative-intent liver resection at the University Clinic for Digestive Surgery, Clinical Center of Serbia, Belgrade, Serbia. The subjects were included in the study if their age range was 18–90 and they had been treated for potentially curative hepatectomy for CRLM, simultaneously or after radical resection of a primary tumor. The exclusion criteria were previous hepatectomy for CRLM and the presence of residual extrahepatic disease. The study was approved by the Ethics Committee of the Clinical Center of Serbia. Informed consent was obtained from all individual participants included in the study. The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and its later amendments or comparable ethical standards.

Demographic and clinicopathological features recorded were age, sex, primary cancer data (localization, neoadjuvant/adjuvant chemo-radiotherapy, TNM, and Duke's classification), metastatic cancer data (neoadjuvant/adjuvant chemo-radiotherapy, number, diameter, and lobar distribution of liver metastases, presence of metastasis at diagnosis), carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, perioperative chemotherapy, tumor grading, and tumor residual status. Preoperative tumor staging was determined by transabdominal ultrasound, chest radiography, computed tomography (CT), and/or magnetic resonance imaging (MRI).

All patients were followed up for one year after surgery. Different diagnostic tests were performed at several time points in order to detect the possible recurrence of the disease. These tests included: measurement of the tumor markers CEA and CA 19-9 and transabdominal ultrasonography (every three months), CT and/or MRI (six months and one year after liver resection), while colonoscopy/rectoscopy and positron emission tomography were considered if local or distant recurrence was suspected. Early recurrence was defined as the presence of either recurrence at the site of primary cancer resection, appearance of new liver metastases, or extrahepatic disease one-year postoperation. Patients were further stratified into recurrence and no recurrence group in regard to the status of recurrent disease.

Samples from CRLM patients. In total, 105 samples were collected from 35 CRLM patients. CRLM and surrounding non-tumor liver tissue samples were obtained from resected liver immediately after surgery. Samples were straightaway immersed into RNA*later*® RNA Stabilization Solution (Thermo Fisher Scientific, Lithuania) and stored at −80 °C. Patients' whole blood samples were obtained one week before surgery. Whole blood samples were allowed to coagulate for 15 min at room temperature and then centrifuged at 3,500×g for 10 min. Obtained sera (supernatant) were aliquoted, once again centrifuged at 3,500×g for 10 min to remove any possibility of contamination with blood cells and stored at −80 °C until RNA extraction.

***In vitro* cultivation and treatments of SW620 and HCEC-1CT cells.** Human metastatic colon adenocarcinoma SW620 cells were grown in 10% fetal bovine serum-supplemented Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, USA). Human colonic epithelial progenitor HCEC-1CT cells were purchased from Evercyte and grown in ColoUp medium (Evercyte, Austria) with 2% fetal bovine serum. Both cell lines were supplemented with 10 U/μl penicillin/streptomycin and cultured in 5% CO_2 humidified air at 37 °C. Cells were subcultured using 1× trypsin/EDTA (Sigma, USA) every third day.

For the MTT assay, 1.5×10^4 SW620 cells and 6×10^3 HCEC-1CT cells were seeded per well in 96-well plates. Cells were left for 24 h to attach and for the next 72 h treated with FOX (21.4 μM 5-fluorouracil (5-FU) (EBEWE Pharma, Austria) and 85 μM oxaliplatin (Actavis, Italy), as reported previously [30] or with 3 clinically relevant concentrations of bevacizumab (Avastin®, Roche, Switzerland) based on its clinical pharmacokinetics analysis: 25 μg/ml (concentration below steady-state), 85 μg/ml (steady-state concentration) [32, 33], and 250 μg/ml (average concentration of maximal plasma concentration reported in Liston et al. [34] and Zhi et al. [33] or with FOX/bevacizumab combinations.

For miR-93-5p expression analysis, 5×10^5 SW620 and 2.5×10^5 HCEC-1CT cells were seeded in 6-well plates and treated on the next day with FOX, 250 μg/ml bevacizumab, or FOX/250 μg/ml bevacizumab combination for 72 h and then lysed and collected with TRIzol reagent (Thermo Fisher Scientific, Lithuania) and stored at –80 °C until RNA extraction.

Generation of 5-FU resistant SW620. To make a clinically relevant model, which mimics the condition of mCRC patients experience during chemotherapy, 5-FU resistant SW620 cells (SW620 5-FUR) were developed by a stepwise increase of 5-FU concentration (5, 10, 15, 20, and 30 μM 5-FU, each treatment lasted 3 days) followed by a recovery period in a drug-free media (4 days). One-week cycle was repeated until the recovered cells formed a confluent layer in a T-75 flask. Cell sensitivity to different 5-FU concentrations (10–300 μM range) after 72 h was checked by MTT and IC_{50} values were determined for SW620 and SW620 5-FUR from 3 experiments. Fold resistance was calculated as IC_{50}–SW620 5-FUR/IC_{50}–SW620. SW620 5-FUR cells were generated during a 6-month period. SW620 5-FUR cells were maintained in 30 μM 5-FU, however, prior to experiments, cells were grown in drug-free media for one passage.

RNA extraction. Collected CRLM and non-tumor liver tissue were cut into smaller pieces and manually homogenized in 2×0.5 ml of TRIzol Reagent. Total RNA from tissue and cell culture was extracted according to the manufacturer's protocol, while the total RNA from serum was extracted using the protocol as previously described [35]. The concentration and purity of total RNA isolated from CRLM, liver tissue, and serum were determined by using BioSpec-nano spectrophotometer (Shimadzu, Japan).

miRNA expression analysis. Fifty nanograms (ng) of total RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and using the following program: 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C.

Tissue, circulating and miR-93-5p expressed in cell lines were detected using TaqMan MicroRNA Assay ID 001090 (Thermo Fisher Scientific, USA), while miR-16-5p (Assay ID 001093) was used as endogenous control. In this study, miR-16-5p was used as a normalizer because it is highly expressed [36] and relatively stable in human cancer cell lines [37], various tumor samples, including CRC [38] and serum [39, 40]. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) reactions were prepared by following the original protocol and run on a 7500 Real-Time PCR System (Applied Biosystems, USA). PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Data were extracted by 7500 System Software and analyzed by $2^{\Delta Ct}$ (patients) or $2^{-\Delta\Delta Ct}$ method (cell culture). Each sample was run in triplicates. The criterion for reproducibility was set to 0.5 cycles [41]. This means that results for the biological sample were discarded or repeated when the Ct values of replicate reactions differed more than 0.5 cycles.

MTT assay. The viability of SW620 and HCEC-1CT cells was evaluated using the MTT assay. Cells were seeded and treated as described above. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) powder was dissolved in PBS. MTT solution was diluted in a medium in a final concentration of 0.5 mg/ml and was added to each well and incubated for 2 h at 37 °C in the incubator. Afterward, the medium with MTT was discarded and cells were lysed in 100 μl DMSO for 30 min. The absorbance was measured at 550 nm on the Infinite M200 PRO plate reader (TECAN, Switzerland). Data were analyzed using Magellan 7.2 software. Cells that were not incubated in MTT solution served as the blank. The percentage of cell survival was calculated using absorbance values (Abs) with the following formula: % of viable cells = $((Abs_{sample}-Abs_{blank})/(Abs_{control}-Abs_{blank})) \times 100$. Each experiment was repeated 3 times.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 9.0.0 (California, USA) and IBM SPSS Statistics 20.0 (Illinois, USA). Categorical variables were expressed as absolute numbers (percentages) while continuous variables were expressed as median (range) or mean ± standard deviation (SD). In this study error bars (SD) are presented when there was a biological variation between treatments or groups and are not shown for technical replicates. Normal distribution was tested using Kolmogorov-Smirnov normality test. In the case of normally distributed data, Student's t-test or one-way ANOVA followed by Dunette's post hoc test was applied, and Pearson bivariate correlation coefficient was determined. Kruskal-Wallis's test, Mann-Whitney U test for two independent samples, or Wilcoxon signed-rank test were used whenever appropriate for non-normally distributed data. Also, nonparametric Spearman's rank correlation was determined for non-normal distributions. A p-value ≤0.05 was considered statistically significant.

Receiver operating characteristic (ROC) curves were created to determinate sample-based cut-off values of miR-93-5p expression in CRLM and serum by Youden index (Youden index = sensitivity + specificity – 1, larger test results indicate a more positive test for CRLM and serum miR-93-5p expression). Patients were stratified into the appropriate

high-miR-93-5p and low-miR-93-5p groups based on the determined cut-off values. Kaplan-Meier method was used to estimate one-year recurrence-free survival of the high- and low- miR-93-5p groups while the comparison was done using Log-rank test. Bivariate analysis was performed using Fisher's exact test for categorical variables to analyze prognostic factors affecting early recurrence.

Results

Ex vivo analysis

Study subjects. The clinicopathological characteristics of the primary and metastatic tumor of patients with CRLM included in this study are given in Table 1.

Regarding administration of neoadjuvant chemotherapeutic treatment for CRLM, 17 patients received 5-FU-based therapy in the form of FOLFOX-bevacizumab (n=12), CAPOX-bevacizumab (n=3), FOLFOX-CAPOX (n=1), Capecitabine (n=1), while one patient received cetuximab-irinotecan as a second line therapy after first-line FOLFOX-bevacizumab treatment. Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 guidelines were employed to evaluate objective tumor response after neoadjuvant chemotherapy for CRLM [42]. In brief, patients were first classified into complete response (CR, disappearance of all target lesions), partial response (PR, at least a 30% decrease in the sum of diameters of target lesions), progressive disease (PD, at least a 20% increase in the sum of diameters of target lesions), and stable disease groups (SD, neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease). Patients were further classified into responders and non-responders. Responders included patients with CR and PR whereas non-responders included patients with SD or PD [43].

miR-93-5p expression in CRLM and non-metastatic liver. The miR-93-5p expression was evaluated in CRLM and non-metastatic liver of patients with mCRC. The expression of miR-93-5p was higher in 31/35 (88.6%) patients, while 4/35 (11.4%) patients had lower miR-93-5p expression in comparison to the non-metastatic liver tissue (Figure 1A). As it can be observed in Figure 1B, the relative miR-93-5p expression was significantly higher in CRLM in comparison to the non-metastatic liver ($p<0.001$).

Estimation of cut-off values from ROC curve analysis. To stratify the patients into miR-93-5p high and low groups, Receiver Operating Characteristic (ROC) curve analysis and Youden's index were employed and sample-based cut-off values for CRLM and serum miR-93-5p expression were estimated. An optimal cut-off value for CRLM and serum miR-93-5p expression was 0.27 (sensitivity 62.5%, specificity 57.9%) and 0.04 (sensitivity 86.7%, specificity 47.4%), respectively. Patients were then divided into the high miR-93-5p expression group (higher miR-93-5p expression than the cut-off value) and the low miR-93-5p expression group (lower miR-93-5p expression than the cut-off value). Regarding miR-93-5p expression in CRLM, 18 (51.4%) patients had high miR-93-5p expression and 17 (48.6%) had low miR-93-5p expression, while in serum 23 (67.7%) patients had high miR-93-5p expression and 11 (32.3%) had low miR-93-5p expression.

Association of CRLM and serum miR-93-5p expression with clinicopathological characteristics of CRLM patients. To determine whether CRLM and serum levels of miR-93-5p were associated with clinicopathological characteristics, the CRLM patients were stratified by age and gender, number, maximal diameter, presence, lobar distribution and presentation of metastases, histological grade, residual status, and recurrence. There was no statistically significant association between miR-93-5p expression and clinicopathological characteristics of CRLM patients ($p>0.05$ for all).

Correlation of miR-93-5p expression in CRLM with its serum expression and tumor markers. To determine whether miR-93-5p expression in CRLM correlates with the serum miR-93-5p expression, Spearman's rank correlation coefficient was calculated. As shown in Figure 2A, there was no significant correlation between CRLM and serum miR-93-5p expression (Spearman's rho (r)=0.166; p=0.349). The correlation between miR-93-5p expression in CRLM

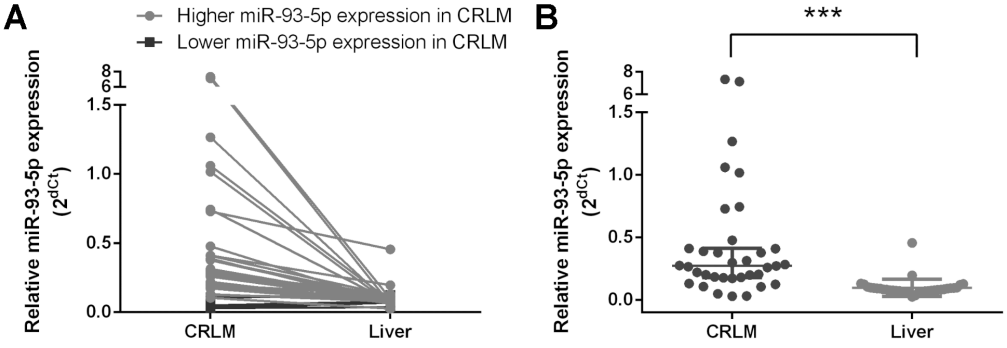

Figure 1. The expression of miR-93-5p in CRLM and non-metastatic liver of patients with mCRC. Relative expression levels (2ΔCt) of miR-93-5p in CRLM and matched non-metastatic liver of mCRC patients shown as paired samples plot (A) and scatter plot (B). ***p<0.001

Table 1. The clinicopathological characteristics of patients with CRLM.

	Study subjects (n=35)
Age (years), mean±SD (range)	63.7±12.2 (24–84)
Sex, N (%)	
male	19 (54.3)
female	16 (45.7)
Tumor markers	
CEA (ng/ml), mean ± SD (range)	57.4±121.3 (2–704)
CA 19-9 (IU/ml), mean ± SD (range)	669.7±2267.2 (2–12999)
Primary cancer information	**n (%)**
Site	
Colon	14 (40.0)
Rectum	21 (60.0)
T stadium	
T1	1 (2.9)
T3	24 (68.6)
T4	10 (28.6)
N stadium	
N0	8 (22.9)
N1	16 (45.7)
N2	11 (31.4)
L stadium	
L0	10 (28.6)
L1	25 (71.4)
V stadium	
V0	8 (22.9)
V1	27 (77.1)
R stadium	
R0	30 (85.7)
R1	5 (14.3)
PN stadium	
PN0	16 (45.7)
PN1	19 (54.3)
Duke's classification	
A–B	3 (8.6)
C–D	32 (91.4)
Therapy	
Neoadjuvant chemoradiotherapy	
No	29 (82.9)
Yes	6 (17.1)
Adjuvant chemotherapy	
No	28 (80.0)
Yes	7 (20.0)
CRLM information	**n (%)**
Number	
≤3	23 (65.7)
>3	12 (34.3)
Maximal diameter	
≤5 cm	27 (77.1)
>5 cm	8 (22.9)
Lobar distribution	
Unilobar	20 (57.1)
Bilobar	15 (42.9)
Presentation	
Synchronous	25 (71.4)
Metachronous	10 (28.6)
Presence	
Solitary	15 (42.9)
Multiple	20 (57.1)
Histologic grade	
Well differentiated (G1)	16 (45.7)
Moderately differentiated (G2)	19 (54.3)
Residual status	
R0	10 (28.6)
R1	25 (71.4)
Therapy	
Neoadjuvant chemotherapy	
No	18 (51.4)
Yes	17 (48.6)
Adjuvant chemotherapy	
No	16 (45.7)
Yes	19 (54.3)

Abbreviations: n-number of patients, SD-standard deviation, T-tumor, N-node, L-lymphatic vessels invasion, V-vein invasion, R-resection, PN-perineural invasion, CRLM-colorectal liver metastasis, CEA-carcinoembryonic antigen, CA-carbohydrate antigen

or serum with tumor markers CEA and CA 19-9 was also analyzed. There was statistically significant moderate negative correlation between CRLM miR-93-5p expression and CEA levels (r=–0.406; p=0.016, Figure 2B). However, there was no significant correlation between serum miR-93-5p expression and CEA (r=–0.112; p=0.528) or CRLM and serum miR-93-5p expression and CA 19-9 levels (r=–0.067; p=0.703 and r=–0.187; p=0.291, respectively).

Predictive potential of miR-93-5p. Out of 35 patients, 17 (48.6%) received neoadjuvant chemotherapy for metastatic disease. To explore whether administration of neoadjuvant chemotherapy has an effect on miR-93-5p expression, we compared CRLM and serum miR-93-5p expression in patients who received neoadjuvant chemotherapy with those who have not, and there was no statistical significance (p>0.05). Based on RECIST criteria, 10 patients (58.8%) had PR, 6 (35.3%) SD, and 1 (5.9%) PD, thus there were 10 responders and 7 non-responder patients. To evaluate the predictive value of miR-93-5p, it was analyzed whether high or low miR-93-5p expression in CRLM and serum corresponds to the patient's therapy response. However, there was no statistically significant association between high-/low-miR-93-5p expression in CRLM or serum and whether the patient responded or not to therapy (p>0.05).

Prognostic potential of miR-93-5p for recurrence-free survival. Kaplan-Meier method was used to estimate

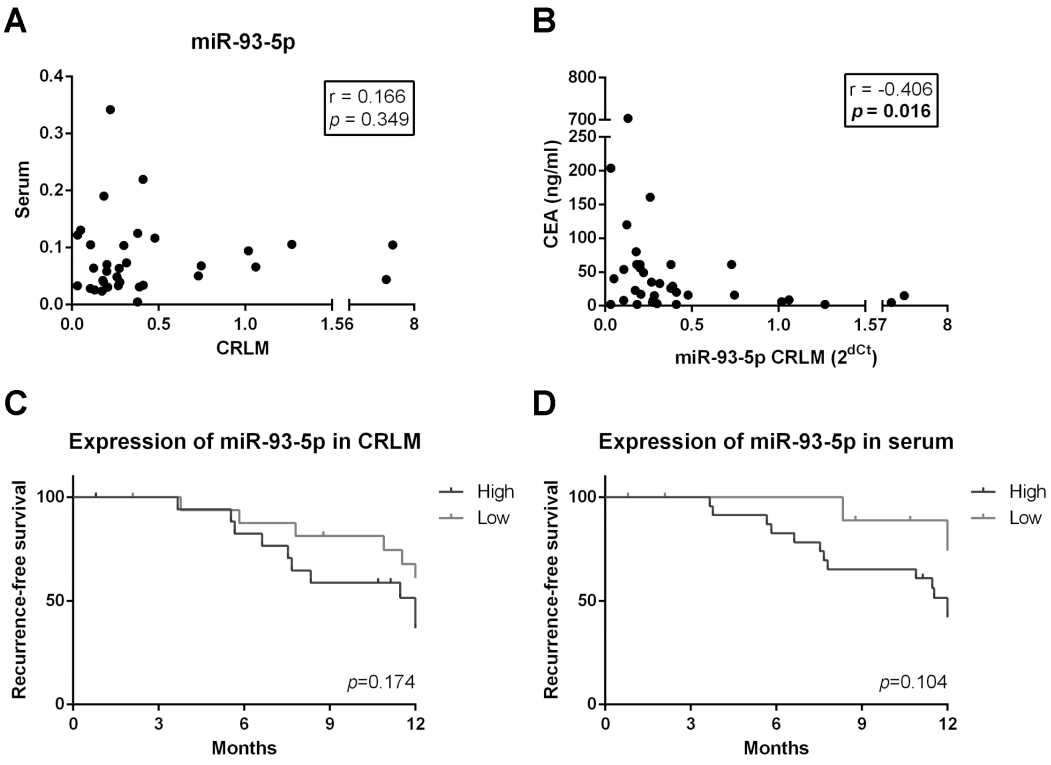

Figure 2. Expression of miR-93-5p in CRLM and serum – correlation with tumor markers and Kaplan-Meier analysis. Scatter plot representation of correlation of miR-93-5p expression in CRLM and serum (A), and miR-93-5p expression in CRLM and CEA tumor markers levels (B). Kaplan-Meier curves of one-year recurrence-free survival for high- and low- miR-93-5p groups based on CRLM (C) and serum (D) expression. Abbreviation: r- Spearman's rank correlation coefficient

one-year recurrence-free survival of the high- and low-miR-93-5p groups. There were no differences in one-year recurrence-free survival of patients with high miR-93-5p expression in CRLM and serum than in patients with low miR-93-5p expression (p=0.174 and p=0.104, respectively, Figures 2C, 2D).

Prognostic potential of miR-93-5p and clinicopathological characteristics of CRLM patients for early recurrence. Bivariate analysis was performed to evaluate the prognostic significance of miR-93-5p and clinicopathological characteristics of CRLM patients for early recurrence. As shown in Table 2, only serum miR-93-5p level was significantly associated with early recurrence, specifically, higher miR-93-5p expression was associated with more patients with recurrent disease, p=0.035). Since serum miR-93-5p level was the only prognostic factor associated with recurrence, multivariate analysis was not performed.

***In vitro* analysis**

To study the effect of systemic, targeted, and combinational therapy for CRLM on normal and metastatic CRC cells and on miR-93-5p expression in more detail, and to confirm the clinical findings, *in vitro* experiments were performed. Since the majority of CRLM patients (15 out of 17) received neoadjuvant treatment in the form of a 5-FU/oxaliplatin/bevacizumab combination, these drugs were used *in vitro*.

Effects of FOX alone or in combination with bevacizumab on the viability of SW620 and HCEC-1CT cells. To study the effects of systemic, targeted, and combination of systemic and targeted therapy for CRLM patients *in vitro*, SW620 and HCEC-1CT cells were treated with FOX, 25, 85, and 250 μg/ml bevacizumab or with a FOX/bevacizumab combination, respectively and the cell survival was analyzed using MTT assay. As it can be observed from Figures 3A and 3B, FOX treatment alone was able to reduce the cell survival of SW620 to 78.0±11.0% (p=0.029) and of HCEC-1CT cells to 71.1±4.9% (p<0.001). Increasing concentrations of bevacizumab had no effect on SW620 viability, whereas the viability of HCEC-1CT cells was reduced to 84.4±2.9% by 85 μg/ml bevacizumab (p=0.033) and to 87.4±9.3% by 250 μg/ml bevacizumab (p=0.048). As for combined FOX/bevacizumab treatments, only FOX/250 μg/ml bevacizumab was able to significantly reduce the viability of SW620 cells to 47.6±3.1% (p=0.003), while FOX/85 μg/ml bevacizumab and FOX/250 μg/ml bevacizumab reduced the viability of HCEC-1CT cells to 40.6±10.1% (p=0.003) and 37.9±6.4% (p=0.002), respectively.

miR-93-5p expression in response to FOX alone or in combination with bevacizumab in SW620 and HCEC-1CT cells. It was already shown that neoadjuvant chemotherapy does not affect CRLM miR-93-5p levels (section Predictive potential of miR-93-5p) in mCRC patients, thus an *in vitro* experiment was set up to confirm this finding.

Basal miR-93-5p expression was determined in SW620 and HCEC-1CT cells cultured for 72 h. miR-93-5p expression was higher in SW620 cells in comparison to the HCEC-1CT cells ($p<0.001$, Figure 3C). Since only the highest tested concentration of bevacizumab (250 μg/ml) in combination with FOX was able to significantly reduce the viability of both SW620 and HCEC-1CT cells (Figures 3A, 3B), this concentration was used to analyze if the bevacizumab alone, or in combination with FOX, affects miR-93-5p expression. As shown in Figure 3D, FOX, 250 μg/ml bevacizumab nor FOX/250 μg/ml bevacizumab combination had an effect on miR-93-5p expression ($p>0.05$) in SW620 cells. However, in HCEC-1CT cells (Figure 3E), FOX decreased the miR-93-5p expression by 22.8±9.2% ($p<0.008$), as well as a FOX/250 μg/ml bevacizumab combination by 23.7±5.3% ($p<0.006$), while the 250 μg/ml bevacizumab had no effect on miR-93-5p expression ($p=0.79$).

Expression of miR-93-5p in SW620 5-FU resistant cells. All of our study group subjects have received some form of 5-FU-based chemotherapy for CRLM. Patient data showed that there was no statistically significant association between miR-93-5p expression and therapy responders vs. non-responders. To confirm this, we have made an *in vitro* 5-FU-resistant mCRC model.

To generate 5-FU resistant SW620 cells, parental SW620 cells were treated with increasing concentrations of 5-FU ranging from 5 to 30 μM. After 6 months, the obtained cell sensitivity was checked by MTT and dose-response curves were generated (Figure 3F), and fold resistance was calculated based on IC_{50} values of parental and 5-FU resistant SW620 cells. The average IC_{50} of parental SW620 was 196.34 μM, whereas the average IC_{50} of SW620 5-FU resistant cells was 351.11 μM. Fold resistance was approximately 1.8.

To analyze if miR-93-5p is associated with resistance to 5-FU, miR-93-5p expression was measured in 5-FU resistant SW620 cells. As it can be observed in Figure 3G, there was no statistically significant difference in miR-93-5p expression in the parental SW620 cells in comparison to the 5-FU resistant SW620 cells.

Table 2. Bivariate analysis of potential prognostic factors for early recurrence in patients with CRLM (N=35).

Characteristics of mCRC patients and CRLM	No Recurrence N=19 (54.3%)	Recurrence N=16 (45.7%)*	p-value
Age			
≤65	7 (36.84%)	7 (43.75%)	0.739
>65	12 (63.16%)	9 (56.25%)	
Gender			
male	10 (52.63%)	9 (56.25%)	1.000
female	9 (47.37%)	7 (43.75%)	
Number of metastases			
≤3	12 (63.16%)	11 (68.75%)	1.000
>3	7 (36.84%)	5 (31.25%)	
Maximal diameter			
≤5 cm	14 (73.68%)	13 (81.25%)	0.700
>5 cm	5 (26.32%)	3 (18.75%)	
Presence			
solitary	9 (47.37%)	6 (37.5%)	0.734
multiple	10 (52.63%)	10 (62.5%)	
Lobar distribution			
unilobar	11 (57.9%)	9 (56.25%)	1.000
bilobar	8 (42.1%)	7 (43.75%)	
Presentation			
synchronous	11 (57.9%)	14 (87.5%)	0.071
metachronous	8 (42.1%)	2 (12.5%)	
Histologic grade			
G1	11 (57.9%)	5 (31.25%)	0.176
G2	8 (42.1%)	11 (68.75%)	
Residual status			
R0	5 (26.32%)	5 (31.25%)	1.000
R1	14 (73.68%)	11 (68.75%)	
Sidedness of the primary tumor			
left	15 (78.95%)	13 (81.25%)	1.000
right	4 (21.05%)	3 (18.75%)	
miR-93-5p in CRLM			
low	11 (57.9%)	6 (37.5%)	0.315
high	8 (42.1%)	10 (62.5%)	
miR-93-5p in serum			
low	9 (47.37%)	2 (13.33%)	**0.035**
high	10 (52.63%)	13 (86.67%)	

Note: *except for miR-93-5p in serum recurrence group N=15

Discussion

The predictive and prognostic role of miR-93-5p in mCRC has not been studied before. This study aimed to examine the predictive and prognostic potential of miR-93-5p expressed in CRLM and circulating miR-93-5p in serum in the same patients with CRLM.

To analyze the clinical significance of miR-93-5p in mCRC, miR-93-5p expression was evaluated in 35 paired samples of CRLM and non-metastatic liver tissues of CRLM patients. The relative miR-93-5p expression was significantly higher in CRLM in comparison to the non-metastatic liver. To interpret this result, it is necessary to compare desired miRNA expression between the normal colon and normal liver tissue [44]. Expression of miR-93-5p was also higher in the normal colon in comparison to the normal liver tissue [45] thus no new information about miR-93-5p involvement in the metastatic process was observed. Another study reported higher miR-93-5p levels in CRLM, but the comparison was done with respect to the normal colon tissue [46]. Association of CRLM and serum miR-93-5p expression

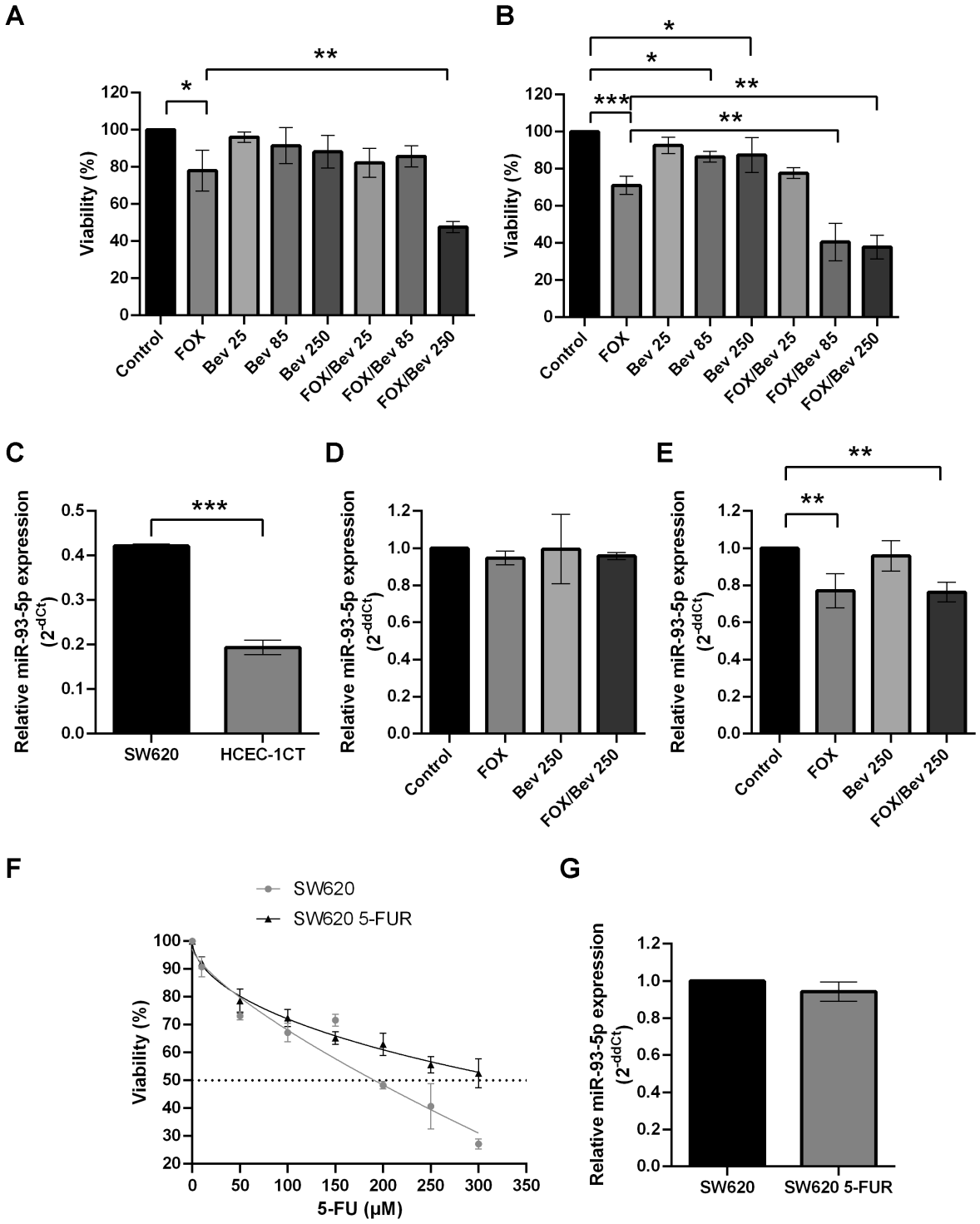

Figure 3. Viability of SW620 and HCEC-1CT cells and miR-93-5p expression in basal state and under treatments. Treatment of SW620 (A, D) and HCEC-1CT (B, E) with FOX, bevacizumab, or with a FOX/bevacizumab combination for 72 h and analysis of cell viability (%) by MTT assay (A, B) and relative miR-93-5p expression (2–ΔΔCt) by qRT-PCR (D, E). In one-way ANOVA analysis (A, B), bev 25, bev 85, and bev 250 treatments were compared to control treatment, while FOX/bev 25, FOX/bev 85, and FOX/bev 250 were compared to FOX treatment. C) Basal miR-93-5p expression in SW620 and HCEC-1CT cells cultured for 72 h. F) Sensitivity of parental and 5-FU resistant SW620 cells to 5-FU. Parental and 5-FU resistant SW620 cells were treated with 0, 10, 50, 100, 150, 200, 250, and 300 μM 5-FU for 72 h and cell viability (%) was analyzed by MTT. G) Relative miR-93-5p expression (2–ΔΔCt) was measured by qRT-PCR in parental and 5-FU resistant SW620 cells. Data are shown as mean ± standard deviation. *p<0.050; **p<0.010; ***p<0.001. Abbreviations: bev 25-bevacizumab 25 μg/ml; bev 85-bevacizumab 85 μg/ml; bev 250-bevacizumab 250 μg/ml, SW620 5-FUR-SW620 5-FU resistant cells

with clinicopathological characteristics of CRLM patients was also analyzed, and no association was found. However, in CRC patients low miR-93 expression was significantly correlated with advanced tumor stage and positive nodal and distant metastases [25], and with the poor tumor differentiation, presence of lymphatic metastasis, and higher TNM and Duke's stages [26].

miRNAs are released from cells into body fluids, and it is possible to detect them as stable circulating molecules in the blood [47]. The circulating miRNAs have been highlighted by numerous studies as a promising non-invasive tool for early detection, prognosis, and therapy selection of CRC patients [48]. There is a limited number of studies, which look for correlation of CRLM and serum miRNA expression in the same patients [49], although this approach could suggest if the changes in circulating miRNA patterns are derived from the tumoral mass or some other source [50]. To explore this, CRLM miR-93-5p levels were correlated with its serum expression, however, no significant correlation was found. CEA and CA 19-9 are well-established tumor markers for detection of disease recurrence, while the combination of these tumor markers with miRNAs could improve the discrimination between patients with favorable and unfavorable outcomes [47]. Thus, we correlated CRLM and serum miR-93-5p levels with tumor markers, and a moderate negative correlation between CRLM miR-93-5p expression and CEA levels was observed. Further studies should clarify the potential benefit of combining standard tumor markers, such as CEA, with miR-93-5p for better prognosis estimation.

miRNAs have been reported as promising tissues and blood biomarkers for the prediction of response to systemic and targeted therapy in CRC patients [51]. Our previous study showed that miR-93-5p was downregulated long-term under 5-FU, oxaliplatin, irinotecan, and 5-FU/oxaliplatin and 5-FU/irinotecan combinations in SW620 cells [30]. Hence, we first examined if the CRLM and serum miR-93-5p expression was altered in patients who received 5-FU-based neoadjuvant chemotherapy with regard to those who have not. Since we did not observe a significant difference, it can be concluded that 5-FU-based neoadjuvant chemotherapy does not affect CRLM and serum miR-93-5p expression in mCRC patients. Next, the predictive value of miR-93-5p was evaluated in patients with CRLM. No significant association was observed between high-/low-miR-93-5p expression in CRLM or serum between therapy responders and non-responders. This was in concordance with the study by Rasmussen et al. involving mCRC patients receiving XELOX/FOLFOX as first-line treatment [52]. Hence, we would argue that miR-93-5p does not have predictive value in CRLM patients treated with 5-FU- or oxaliplatin-based chemotherapy.

miRNAs have also been associated with prognosis and disease recurrence in CRC patients [22, 51]. The lower miR-93-5p expression has been previously associated with early relapse and worse overall and disease-free survival in CRC patients [26, 27, 31]. Here, we have shown that there were no differences in one-year recurrence-free survival of patients with high miR-93-5p expression in CRLM and serum than patients with low miR-93-5p expression, however, high miR-93-5p serum levels were significantly associated with disease recurrence in CRLM patients. Hence, miR-93-5p serum expression could be potentially used as a prognostic factor for early disease recurrence but not for recurrence-free survival.

Several studies have been published previously which investigated the role of miR-93-5p on CRC tumorigenesis, as well as on migratory and invasive properties of CRC cell lines. The tumor-suppressive role of miR-93-5p was shown on proliferation, cell cycle regulation and apoptosis, migration, invasion, and tumor growth *in vivo*. Overexpression of miR-93-5p significantly inhibited cell proliferation and colony formation of SW1116 human colon cancer stem cells grown in the media without serum [53], SW480 and HCT116 cells by regulating the Wnt/β-catenin signaling pathway [54, 55], as well as of LOVO and SW480 cells through the miR-93-5p/HMGB3 regulatory axis [56], and Caco2 cells by regulating the expression of ERBB2, p21, and VEGF [27]. Functional studies showed that increased miR-93-5p expression promotes apoptosis of SW480 and HCT116 cells [55], SW480 and LOVO cells [56], and leads to the G2 phase accumulation of Caco2 cells [27]. Additionally, increased expression of miR-93-5p inhibited migration, but not invasion of Caco2 cells [27], suppressed migration of HCT116 cells [54], suppressed migration and invasion of SW480 and HCT116 cells [26], and LOVO and SW480 cells [56]. In line with the *in vitro* studies, miR-93-5p had a tumor-suppressor role on tumor growth *in vivo*. Mice injected subcutaneously with cells or miR-93-5p overexpression vectors had significantly smaller tumors in comparison to the control group [54, 27]. Probably because these *in vivo* studies found a tumor-suppressive role of miR-93-5p, the precise effect of the increased miR-93-5p expression on CRLM was not presented. Considering our results of the potential prognostic function of circulating miR-93-5p in CRLM patients, further studies are needed to fully elucidate the role of miR-93-5p in colorectal cancer liver metastases.

To confirm the clinical findings, *in vitro* experiments were performed. The effect of systemic (FOX), targeted (bevacizumab), and combinational therapy (FOX/bevacizumab) for mCRC on the viability of normal and metastatic CRC cells and on miR-93-5p expression was studied in more detail because approximately 90% of study subjects who received neoadjuvant treatment, a combination of 5-FU/oxaliplatin/bevacizumab was administered. FOX treatment was able to reduce the cell survival of SW620 and HCEC-1CT cells. The viability of HCEC-1CT cells was significantly dose-dependently reduced under bevacizumab and FOX/bevacizumab treatments, while only the highest tested concentration of bevacizumab in combination with FOX was able to significantly reduce the viability of SW620 cells. In contrast

to our results, Vuletic et al. showed that 25 and 50 μg/ml bevacizumab decreased significantly SW620 cell viability to approximately 90%, whereas we were unable to observe cytotoxic effects even under 250 μg/ml bevacizumab [57]. Another study showed that 250 μg/ml bevacizumab had slightly increased proliferation of SW620, however, these cells were grown in hypoxic (1% O_2) and serum-reduced (1% FBS) conditions [58]. Overall, HCEC-1CT cells were more sensitive to the tested drugs in comparison to the SW620 cells. It is not unusual for an anti-cancer drug to kill normal cells because it is known that 5-FU acts by interfering with DNA and RNA synthesis in both normal and tumor cells [59]. Although there is no literature data about the viability of HCEC-1CT cells treated with 5-FU, oxaliplatin, and bevacizumab, it was shown that 5-FU had a strong cytotoxic effect on CCD112, another normal colorectal cell line [60].

The basal miR-93-5p expression was higher in metastatic SW620 cells in comparison to normal HCEC-1CT cells. In contrast, Tang et al. observed lower miR-93-5p expression in SW620 cells but in comparison to the mean miR-93-5p expression level of normal colonic mucosa from 45 subjects [54]. We have shown that neoadjuvant chemotherapy does not affect CRLM and serum miR-93-5p levels in patients with CRLM and to confirm this finding, an *in vitro* experiment was set up. The effect of a targeted therapy agent bevacizumab on miR-93-5p expression has not yet been studied *in vitro* on the mCRC cell line model. We have shown that 250 μg/ml bevacizumab alone or in combination with FOX does not influence miR-93-5p expression in SW620 cells. In this study, miR-93-5p expression was stable even under FOX, which is in contrast to our previous findings where miR-93-5p expression was downregulated after 72 h of treatment with FOX in the same cell line [30]. The only difference in the two experimental settings was the endogenous control used, RNU6B, which was used in the previous study, and miR-16-5p which was used here, although both endogenous controls have been previously reported to have stable expression and were used for miRNA's expression normalization in SW620 cells [61, 37]. As in SW620 cells, bevacizumab did not have an effect on miR-93-5p expression in HCEC-1CT cells.

Patient data presented here showed that there was no statistically significant association between miR-93-5p expression and therapy responders vs. non-responders. To confirm this, we have made an *in vitro* 5-FU-resistant mCRC model since all of our study group subjects have received some form of 5-FU-based chemotherapy for CRLM. SW620 cells were known to have the highest tolerance to 5-FU in comparison to other CRC cell lines SW480, HCT116, HCT15, HCT8, HT-29, and LOVO cells [62]. Wide range of 5-FU IC_{50} values has been reported for SW620 cells, from as low as 0.5 μM [63], 8–25 μM [64–67] to high as 100 μM [68, 69]. We found a 5-FU IC_{50} value of 196.34 μM which is higher in comparison to the literature data, however, it should be mentioned that these studies differ significantly in the treatment duration (from 24 h to 120 h) and assay used to measure cell viability (MTT, Cell Counting Kit-8 assay, and sulforhodamine assay). Generated SW620 5-FU resistant cells exhibited 1.8 5-FU fold resistance in comparison to the parental SW620 cells, which is close to the lower limit for clinically relevant fold resistance of 2 [70]. It was shown that miR-93-5p expression was similar in parental SW620 cells in comparison to the 5-FU resistant SW620 cells, thus confirming the lack of miR-93-5p chemosensitivity to 5-FU. Overall, *in vitro* data confirmed that miR-93-5p cannot be used as a predictive biomarker for CRLM therapy response follow-up.

The major limitation of this study is the relatively small sample size. Although 3 different samples were collected from each of the 35 patients with CRLM, the sample size could limit the power of statistical analysis, so further studies involving more CRLM patients are warranted in order to validate our preliminary results.

In conclusion, this is the first study to examine the predictive and prognostic value of miR-93-5p in mCRC patients either as tissue or circulating biomarker. We have shown that miR-93-5p could not be used as a predictive biomarker in CRLM patients treated with 5-FU-based chemotherapy, however, since high miR-93-5p serum levels were significantly associated with early disease recurrence, circulating miR-93-5p levels could serve as a prognostic factor for early disease recurrence in CRLM patients after liver resection. Further large-scale studies are warranted to confirm circulating miR-93-5p prognostic capacity.

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