Circulating miRNA expression over the course of colorectal cancer treatment

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Abstract. Colorectal cancer (CRC) is the third-most common cancer type in males and the second-most common cancer type in females, and has the second-highest overall mortality rate worldwide. Approximately 50% of patients in stage I-III develop metastases, mostly localized to the liver. All physiological conditions occurring in the organism are also reflected in the levels of circulating microRNAs (miRNAs/miRs) in patients. miRNAs are a class of small, non-coding, single-stranded RNAs consisting of 18-25 nucleotides, which have important roles in various cellular processes. The aim of the present study was to evaluate a panel of seven circulating miRNAs (miR-106a-5p, miR-210-5p, miR-155-5p, miR-21-5p, miR-103a-3p, miR-191-5p and miR-16-5p) as biomarkers for monitoring patients undergoing adjuvant treatment of CRC. Total RNA was extracted from the plasma of patients with CRC prior to surgery, in the early post-operative period (n=60) and 3 months after surgery (n=14). The levels of the selected circulating miRNAs were measured with the miRCURY LNA miRNA PCR system and fold changes were calculated using the standard ΔΔCq method. DIANA-miRPath analysis was used to evaluate the role of significantly deregulated miRNAs. The results indicated significant upregulation of miR-155-5p, miR-21-5p and miR-191-5p, and downregulation of miR-16-5p directly after the surgery. In paired follow-up samples, the most significant upregulation was detected for miR-106a-5p and miR-16-5p, and the most significant downregulation was for miR-21-5p. Pathway analysis outlined the role of the differentially expressed miRNAs in cancer development, but the same pathways are also involved in wound healing and regeneration of intestinal epithelium. It may be suggested that these processes should also be considered in studies investigating sensitive and easily detectable circulating biomarkers for recurrence in patients.

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Introduction

Colorectal cancer (CRC) is the third-most common cancer type in males and the second-most common cancer type in females, and has the second-highest overall mortality rate worldwide (1). Major risk factors for CRC include a Western dietary pattern, sedentary lifestyle and increasing obesity due to lifestyle factors related to economic growth and industrialization. CRC has a relatively higher incidence in economically developed countries, which may be associated with a higher life expectancy, education and human developmental index due to better diagnosis of CRC (2). Furthermore, preventable lifestyle habits such as smoking, alcohol consumption and obesity contribute to an increased risk of tumour formation (3). Abdominal fat is sub-divided into two compartments: Visceral adipose tissue (VAT) and subcutaneous adipose tissue. VAT is responsible for the secretion of pro-inflammatory adipokines (such as TNF); in addition, it is infiltrated with macrophages, leading to chronic inflammation and promoting tumour growth (4). Furthermore, insulin resistance related to obesity has an important role in the promotion of carcinogenesis. Chronic inflammation is also a condition responsible for the elevated risk of CRC in patients with ulcerative colitis and Crohn's disease (3). In general, cancer is a disease associated with ageing; the risk of CRC markedly increases after the age of years 50. However, a higher incidence in individuals aged <40 years has also been observed (4).

Approximately 50% of patients in stage I-III develop metastases mostly localized to the liver (5); furthermore, ~20% of patients already have liver metastases at the time-point of diagnosis (6). Patients with stage I disease have a 5-year survival rate of 90%; however, a marked decline is observed

in patients with stage IV disease (6). Understanding molecular pathway abnormalities is crucial for improving the diagnosis, prognosis and treatment of CRC, as it is a heterogeneous multifactorial disease with a range of different prognoses and responses to therapy (7). During the last decade, the development of novel targeted therapies and chemotherapeutics has led to improved clinical outcomes for patients with advanced metastatic disease. Despite the current screening methods and prognostic factors, there are still numerous patients that are not profiting from novel treatment strategies (4), which makes the early diagnosis of CRC recurrence key for improving prognosis and reducing mortality. Therefore, there is a significant requirement for a representative, sensitive and easily detectable biomarker (8).

MicroRNAs (miRNAs/miRs) are a class of small, non-coding, single-stranded RNAs consisting of approximately 18-25 nucleotides (9). They regulate the translation of protein-coding genes at the post-transcriptional level by binding to complementary sequences in the 3-untranslated regions of their target mRNAs (10), leading to inhibition of translation or degradation of the mRNA (11). In addition to tumour tissue, miRNAs have been identified in numerous biological sample types, including plasma, serum, saliva, faeces, urine, cerebrospinal and amniotic fluid (12). Circulating miRNAs are actively released into the bloodstream from tumour cells and may be detected in the form of exosomes, free or bound to proteins (11). miRNAs may either function as tumour suppressors through inhibition of oncogene expression or as oncogenic miRNAs by inhibiting the expression of tumour suppressor genes. Due to their localization within fragile sites in the genome, the expression of miRNAs may be dysregulated by different genetic alterations, including deletions, amplifications, translocations and point mutations, and also DNA hyper-methylation and hypo-methylation (10). Altered miRNA expression has been identified in various types of cancer, e.g., pancreatic cancer, hepatocellular cancer, breast cancer, CRC and lung cancer (13). miRNAs have important roles in cellular processes and are also involved in mechanisms of cancer development, such as cell proliferation, differentiation, apoptosis, angiogenesis and epithelial-mesenchymal transition. Several miRNAs have been suggested as candidates for CRC diagnosis; however, it is still difficult to draw clear conclusions (12).

Carcinoembryonic antigen (CEA) is a widely used marker for CRC recurrence detection recommended by the European Group on Tumour Markers and the American Society of Clinical Oncology (14,15). Furthermore, CEA may be detected from peripheral venous blood, which makes CEA a suitable marker; however, its sensitivity is not sufficient in certain cases (16). The plasma level of CEA should be established pre-operatively to monitor the dynamics of its concentration after surgery, which is not always accomplished. In addition, CEA may only be present at low concentrations in patients with poorly differentiated tumours. miRNAs are easily detectable and stable biomarkers; however, further research is required to establish them as reliable biomarkers for local and distant recurrence of CRC. Implementation of miRNAs in clinical practice remains a challenge due to their diversity in affecting numerous molecular and cellular processes (17).

Apart from cancer, miRNAs have important roles in numerous molecular networks associated with inflammatory and autoimmune disorders. There is evidence that a connection exists between cancer and chronic inflammation (18), in which miR-155, miR-210 and miR-21 are involved. It has been indicated that upregulation of miR-155 results from oncogenic and inflammatory stimuli and triggers malignant transformation. Furthermore, miR-155 overexpression is frequently associated with high cytokine production (19). Studies have indicated that miR-21 is involved in both negative and positive feedback loops controlling inflammation. This has been demonstrated in different cell types under different conditions; therefore, this may be an explanation for the opposing effects of miR-21 on inflammation (20). Inflammation itself is linked with wound healing, which, along with systemic inflammatory response syndrome, is considered to be a normal post-operative condition. The pro-inflammatory microenvironment is also present during chemotherapy because of cellular senescence due to the acquired pro-inflammatory senescence-associated secretory phenotype of these cells. The same miRNAs have been indicated to be involved in wound healing, as similar mechanisms and pathways are responsible for inflammation. These miRNAs include the mentioned miR-21 and miR-155 (19), as well as miRNAs involved in angiogenesis, such as miR-16, miR-103, miR-191 and miR-21, the expression of which has been investigated in a study (20).

The aim of the present study was to evaluate a panel of seven circulating miRNAs in pre-operative, post-operative and 3-month follow-up samples to investigate changes in the level of selected miRNAs immediately after surgery and a longer time interval after the operation.

Materials and methods

Patients. Patients with diagnosed CRC or colorectal adenoma who underwent surgical intervention at the Clinic of Surgery and Transplant Centre, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, (Martin, Slovakia) were enrolled in the present study. Patients were informed about the study and signed the informed consent form approved by the Ethical Committee at Jessenius Faculty in Martin, Comenius University in Bratislava (Martin, Slovakia) in accordance with the Declaration of Helsinki. The cohort included 110 patients with CRC or colorectal adenoma selected according to the following criteria: i) All patients were diagnosed with a defined clinical stage; ii) disease was confirmed by routine histopathological examination; and iii) none of the patients had any other disease or known malignancies that may have affected miRNA plasma levels.

Blood sample collection. Blood samples were collected between January 2018 and August 2020. For each patient, peripheral blood samples were collected two or three times and processed as soon as possible, no later than 1 h after collection. Pre-operative and post-operative blood samples were taken one day prior to surgery and 3-7 days after surgery, respectively. Another set of post-operative blood samples was collected during the follow-up period (3 months after surgical intervention). However, the follow-up group did not include blood samples from all patients (n=20). Patients in the

follow-up group did not receive any chemotherapy at the time of sampling.

Blood samples were collected into K_3EDTA tubes and transported 1 h after collection. The blood plasma was immediately separated from 10 ml of whole blood by two-step centrifugation at 850 x g for 10 min at 4˚C and 18,620 x g for 10 min at 4˚C to completely remove components including cell debris, residual platelets and microvesicles. The plasma samples were stored at -80˚C until use.

Quality control of plasma samples, total RNA extraction and reverse transcription (RT). The thawed plasma samples were visually inspected and the presence of free haemoglobin was measured by determining the absorbance at 414 nm by a Nanodrop® 1000 (ThermoFisher Scientific, Inc.). Samples with an absorbance between 0.039 and 0.25 were included in the study. Only patients with pre-operative/post-operative (n=60) and/or follow-up blood samples (n=14) were included in the study. Total RNA including the miRNA fraction was extracted from 200 μl of plasma using an miRNeasy Serum/Plasma Advanced kit (Qiagen GmbH) following the manufacturer's protocol. RNA spike-in controls (RNA Spike-in kit; Qiagen GmbH) were added into the lysis buffer to provide control of RNA extraction. Total RNA was resuspended in 20 μl of RNase-free water and stored at -80˚C until RT. A total of 10 μl of RT reaction mixture contained, in addition to common ingredients (miRCURY LNA RT kit; Qiagen GmbH), 1 μl of total RNA and 0.5 μl of RNA synthetic spike-ins. RT was performed in a thermal cycler for 1 h at 42˚C, followed by inactivation for 5 min at 95˚C. The obtained cDNA was stored at -20˚C until analysis, no longer than 5 weeks, and diluted at a 1:30 ratio prior to analysis.

Measurement of selected miRNAs by RT-quantitative (q) PCR. Based on a review of the literature (4-26), various circulating miRNAs (miR-106a-5p, miR-210-5p, miR-155-5p, miR-21-5p, miR-103a-3p, miR-191-5p, miR-16-5p) were selected and their levels were analysed. Quantification of each miRNA was performed in duplicate using an miRCURY LNA miRNA PCR System (Qiagen GmbH). All pipetting steps were performed on a Bravo liquid handling station (Agilent Technologies, Inc.) and run in duplicate/triplicate on a LightCycler (LC)480 instrument (Roche Diagnostics GmbH). Rapid quantification analysis was performed using LC480 instrument software and quantification cycle (Cq) values were calculated by the second derivative method (27). Secondary analysis and quality control of RNA isolation and RT through synthetic spike-ins were performed using the GeneGlobe data analysis tool (Qiagen GmbH). The web tool (https://geneglobe.qiagen.com/sk/analyze) was also used for rough data analysis, although no suitable reference genes were found with the geNorm or Normfinder algorithms, as all tested groups had different average arithmetic means.

Statistical analysis. The data were visualized and analysed using R version 3.5.2 (28). Expression and fold changes (FC) were computed using the standard formula (29) for a paired design. Furthermore, normalization of Cq values gene by gene (ΔCq) and between groups is important to minimize the technical variability and the FC is the expression ratio of the miRNA (-$\Delta\Delta$Cq) between two conditions. The null hypothesis that the population median FC is equal to and/or is equal for two levels of a categorical clinical parameter (e.g., sex) was tested by the Wilcoxon rank-sum test. The analogous null hypothesis for a categorical clinical parameter with >2 levels (e.g., Cq) was tested by the Kruskal-Wallis test. The association between two clinical parameters (e.g., sex and Cq) was examined by the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Analysis of involvement of differentially expressed miRNAs in signalling pathways associated with CRC relapse. The involvement of differentially expressed miRNAs (miR-106a-5p, miR-21-5p and miR-16-5p) in signalling pathways associated with CRC relapse was analysed through the online software DIANA mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) based on analysis of functional pathway enrichment, as well as numerous segments of Gene Ontology (GO) analysis in the *Homo sapiens* species (30). The online analysis tool was used for combining the available in silico-predicted targets (DIANA-microT-CDS and/or TargetScan v6.2 algorithm) and high-quality experimentally supported interactions (DIANA-TarBase v7.0 algorithm), and $P<0.05$ was considered to indicate statistical significance. In the KEGG analysis, gene intersections with all three miRNAs included were preferred and in the GO analysis, category intersections for merging the results with false discovery rate correction were chosen.

Results

Patient characteristics. Overall, 110 patients of Caucasian ethnicity with diagnosed CRC or adenoma were recruited for the present study. As indicated in Fig. 1, 30 patients were excluded due to other malignancies or inoperable tumours. The plasma samples of the remaining 80 patients were analysed. Extraction of RNA including the miRNA fraction was performed on 60 samples without haemolysis. If haemolysis was present in the first or second sample taken at different time points, the patient was excluded from analysis. It is known that the circulating miRNA profile may be significantly influenced by haemolysis of red blood cells, which does not mirror the physiological release from cells, including cancer cells (31).

The patient characteristics are presented in Table I. The patients' mean age at the time-point of diagnosis was 69 years [interquartile range (IQR), 63-73 years] for males (n=41) and 68 years (IQR, 58-72 years) for females (n=19). Carcinoma was present in almost 83% of males and 78% of females. Most patients were at stage II or III and in 54% of males and 74% of females, tumours were localized in the proximal colon. Table II contains additional information on neoadjuvant treatment and follow-up. Disease recurrence was present in 40% of males and in 31% of females. Regrettably, it was not possible to acquire follow-up blood samples from 78.1% of males and 73.7% of females due to low compliance of the patients. Furthermore, the situation was complicated by the coronavirus pandemic. The information regarding patient relapse is incomplete, as patients were also managed at other hospitals and there is no national central register in Slovakia.

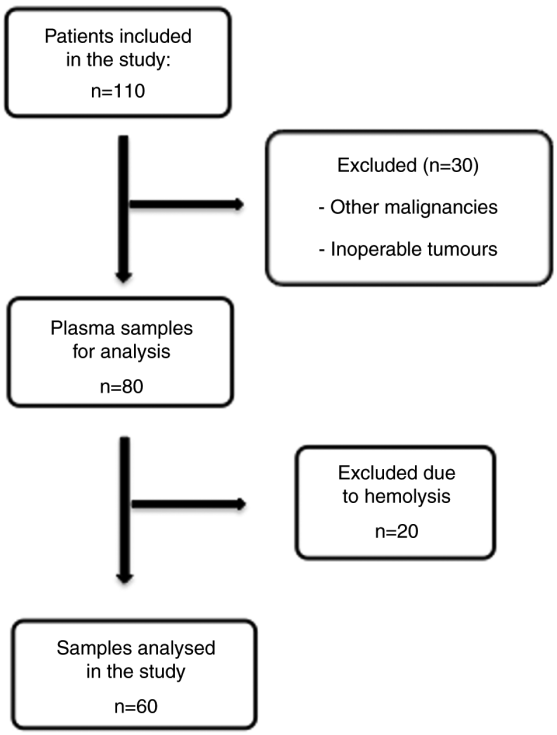

Figure 1. Schematic of patient and sample inclusion and/or exclusion in the study.

Evaluation of Cq values determined by miRNA expression analysis of plasma. Most of the selected miRNAs (miR-106a, -21, -103a, -191 and -16) were detected in plasma samples with calculated Cq values between 22 and 35 using the second derivative method. A small portion of analysed miRNAs (miR-210-5p and miR-155-5p) had Cq>35, which means weak plasma expression and these miRNAs were not included in the general conclusions of the study. Secondary analysis using the GeneGlobe data analysis tool revealed that RNA isolation and RT were performed correctly. During the data normalization process, differences in average arithmetic mean between tested groups from 0.5 to 1 Cq were indicated. No combinations of selected miRNAs were appropriate for data normalization without calculation of the stability factor due to missing Cq values for certain samples. Normalization to the synthetic spike-in *Caenorhabditis elegans* miR-39 (cel-miR-39) was also not appropriate, as it is not affected by the biological condition of the patient and input concentration of miRNA. Therefore, in FC calculations, the median of all measured miRNAs except spike-ins was used, as recommended in the literature (32). In the statistical analysis of FC and the P-value calculation, post-operative and follow-up samples were compared to pre-operative samples.

Circulating miRNA expression in post- vs. pre-operative samples from patients with CRC. The results of the miRNA analysis of samples acquired pre- and post-operatively indicated significant upregulation of miR-21-5p (P=0.010), miR-155-5p (P=0.004) and miR-191-5p (P=0.005), and downregulation of miR-16-5p (P=0.006) (Table III; Fig. 2).

Comparison of miRNA expression with histopathological parameters indicated significant upregulation of miR-210-5p in the circulation of patients who underwent neoadjuvant therapy (P=0.012). When excluding patients with neoadjuvant therapy from the data, miR-106a-5p expression was significantly

Table I. Characteristics of patients included in the study.

Item	Males (n=41)	Females (n=19)
Age, years	69 (63; 73)	68 (58; 72)
Tumor type		
Carcinoma	34 (82.9)	15 (78.0)
Adenoma	2 (4.9)	2 (11.0)
WNL	5 (12.2)	2 (11.0)
Tumor localization		
Rectum, distal colon	19 (46.0)	5 (26.0)
Proximal colon	22 (54.0)	14 (74.0)
Stage		
I	7 (17.1)	2 (10.5)
II	10 (24.4)	3 (15.8)
III	10 (24.4)	9 (47.4)
IV	4 (9.7)	2 (10.5)
Adenomas/polyps	10 (24.4)	3(15.8)
pT		
0	1 (2.4)	0 (0.0)
1	6 (14.6)	0 (0.0)
2	6 (14.6)	2 (10.5)
3	15 (36.6)	8 (42.1)
4	7 (17.2)	4 (21.1)
Adenomas/polyps	6 (14.6)	5 (26.3)
pN		
0	22 (53.7)	7 (36.8)
1	8 (19.5)	3 (15.8)
2	9 (21.9)	4 (21.1)
Unknown	2 (4.9)	5 (26.3)
pM		
0	2 (4.9)	2 (10.5)
1	4 (9.8)	3 (15.8)
x	35 (85.3)	11 (57.9)
Adenomas/polyps	0 (0.0)	3 (15.8)
Grade		
1	14 (34.1)	2 (10.5)
2	12 (29.3)	7 (36.8)
3	4 (9.8)	3 (15.8)
Adenomas/polyps	11 (26.8)	7 (36.8)
Microsatellite stability		
MSS	32 (78.1)	8 (42.1)
MSI	1 (2.4)	5 (26.3)
Adenomas/polyps	8 (19.5)	6 (31.6)
Relapse		
Absent	15 (36.6)	9 (47.4)
Present	10 (24.4)	4 (21.1)
Unknown	16 (39.0)	6 (31.5)

Values are expressed as n (%), except for age, which is expressed as the median (lower quartile; upper quartile). WNL, without neoplastic lesion-patients after neoadjuvant treatment; MSS, microsatellite stable; MSI, microsatellite instable.

Table II. Distribution of male and female patients according to neoadjuvant treatment and 3 months of follow-up.

Item	Males, n (%)	Females, n (%)
Neoadjuvant therapy		
Yes	5 (12.2)	1 (5.3)
No	36 (87.8)	18 (94.7)
Follow-up after 3 m		
Carcinoma	9 (21.9)	4 (21.0)
Adenoma	0 (0.0)	1 (5.3)
No 3 m follow-up	32 (78.1)	14 (73.7)
Relapse		
Absent	15 (36.6)	9 (47.4)
Present	10 (24.4)	4 (21.1)
No information available	16 (39.0)	6 (31.5)

Values are expressed as n (%). 3 m, 3 months.

increased with rising severity of the stage (P=0.033). No association with tumour type, stage, size, nodal status, metastases, grade or relapse was obtained. No significant associations were observed between miR-21-5p, miR-103a-3p, miR-155-5p, miR-191-5p and miR-16-5p expression and histopathological parameters. All P-values are listed in Table SI.

Circulating miRNA expression in plasma of patients with CRC in follow-up samples compared to pre-operative levels. The expression of selected miRNAs in follow-up samples (n=14) was compared to that in pre-operative plasma samples (n=60). The results suggested that miR-106a-5p, miR-191-5p and miR-16-5p were significantly upregulated (P=0.005, 0.048 and 0.003, respectively) in follow-up samples taken 3 months after surgery when compared to the condition before surgery (Fig. 3). However, miR-210-5p (P=0.1309), miR-155-5p (P=0.1099), miR-21-5p (P=0.1353) and miR-103a-3p (P=0.052) did not exhibit any statistically significant changes (Table III).

Statistical analyses were performed to assess any possible associations between miRNA expression and pathological parameters. However, the expression of these seven miRNAs was not statistically significantly associated with the TNM stage, stage, grade or tumour type of the patients (data not shown).

Circulating miRNA expression in paired post-operative and follow-up samples. Changes in the expression levels of the selected panel of miRNAs in paired post-operative and follow-up samples compared to the pre-operative levels are graphically presented in Fig. 4. Analysis of outliers for patients with known histopathological data for each of the selected miRNAs identified follow-up samples from two patients with metastasis in the liver at the time of follow-up sampling with a different profile of miR-210 and miR-21 expression

A summary of the median FCs in paired post-operative and follow-up samples for each investigated miRNA is presented in Table IV. Circulating miR-106a-5p and miR-16-5p were significantly upregulated (P=0.027 and 0.003, respectively) in the follow-up samples and miR-21-5p was significantly downregulated (P=0.035).

Signal and functional pathway analysis. The target genes and function of the most differentially expressed miRNAs associated with tumour removal, wound healing and possible recurrence were analysed by KEGG pathway analysis using the DIANA-mirPath v3.0 online web analysis tool. miR-106a-5p was experimentally proven to have 1,160 target genes, miR-16-5p has 2,886 target genes and miR-21-5p has 1,372 target genes listed in DIANA-TarBase algorithm (accessed, 30 November 2020). In a gene intersection setting for merging the results of the KEGG analysis (Table V), it was indicated that all three miRNAs are significantly enriched in pathways involving proteoglycans in cancer (hsa05205; 9 target genes), the Hippo signalling pathway (hsa04390; 5 target genes), focal adhesion (hsa04510; 11 target genes), signalling pathways regulating pluripotency of stem cells (hsa04550; 7 target genes), the prolactin signalling pathway (hsa04917; 5 target genes), endocrine and other factor-regulated calcium reabsorption (hsa04961; 2 target genes), pathways in cancer (hsa05200: 8 target genes), CRC (hsa05210; 4 target genes), the FoxO signalling pathway (hsa04068; 8 target genes), endometrial cancer (hsa05213; 3 target genes), thyroid cancer (hsa05216; 3 target genes), lysine degradation (hsa00310; 2 target genes) and the p53 signalling pathway (hsa04115; 5 target genes). Three putative target genes (CCND1, CTNNB1 and MAPK1) were indicated to be associated with 6 of the 11 signalling pathways (proteoglycans in cancer, focal adhesion and pathways in cancer, as well as colorectal, endometrial and thyroid cancer).

Investigation of the biological function of target genes regulated by differentially expressed miRNAs in the subcategories biological process, cellular component or molecular function, was performed through GO analysis. GO annotation results for category intersection enrichment of target genes influenced by up- or downregulated miRNAs in patients' follow-up samples are provided in Table VI. The most enriched GO annotations were ‘mitotic cell cycle’ (GO:0000278), ‘otein complex assembly’ (GO:0006461) and ‘cellular protein modification process’ (GO:0006464). The top 10 GO terms in the biological process category were presented and a full list of GO terms is provided in Table SII. A visualization of GO terms in each category is displayed in Fig. 5.

Discussion

Numerous studies have focused on miRNAs and their prognostic and predictive roles in CRC. Various studies had conflicting results regarding the up- and downregulation of selected miRNAs in assessing the prognosis and therapy response. In the present study, a group of seven miRNAs were selected from the literature that were significantly up- or downregulated post-operatively and in follow-up samples when compared to the pre-operative state.

Within the selected panel of circulating miRNAs, the plasma expression of miR-106a-5p was not significantly changed in post-operative samples. However, miR-106a-5p expression was significantly increased in follow-up samples (P=0.005). By contrast, certain studies have demonstrated

Table III. Fold-changes in the levels of selected miRNAs in the post-operative and follow-up samples collected 3 months after surgery compared with pre-operative stages.

miRNA	Post-operative samples (n=60)	P-value	Follow-up samples (n=14)	P-value
miR-106a-5p	1.04 (0.91, 1.18)	0.513	3.48 (1.54, 5.88)	0.005
miR-210-5p	1.47 (0.94, 2.29)	0.086	3.15 (0.8, 12.03)	0.130
miR-155-5p	1.9 (1.26, 3.67)	0.004	3.46 (0.88, 8.24)	0.110
miR-21-5p	1.15 (1.04, 1.3)	0.010	0.65 (0.46, 1.43)	0.140
miR-103a-3p	1.01 (0.83, 1.26)	0.900	0.69 (0.54, 1.04)	0.052
miR-191-5p	1.2 (1.06, 1.37)	0.005	1.48 (1.01, 2.17)	0.048
miR-16-5p	0.83 (0.71, 0.94)	0.006	1.73 (1.16, 2.65)	0.003

Values are expressed as the median (interquartile range). Wilcoxon's rank-sum test was performed for statistical comparisons. miRNA/miR, microRNA.

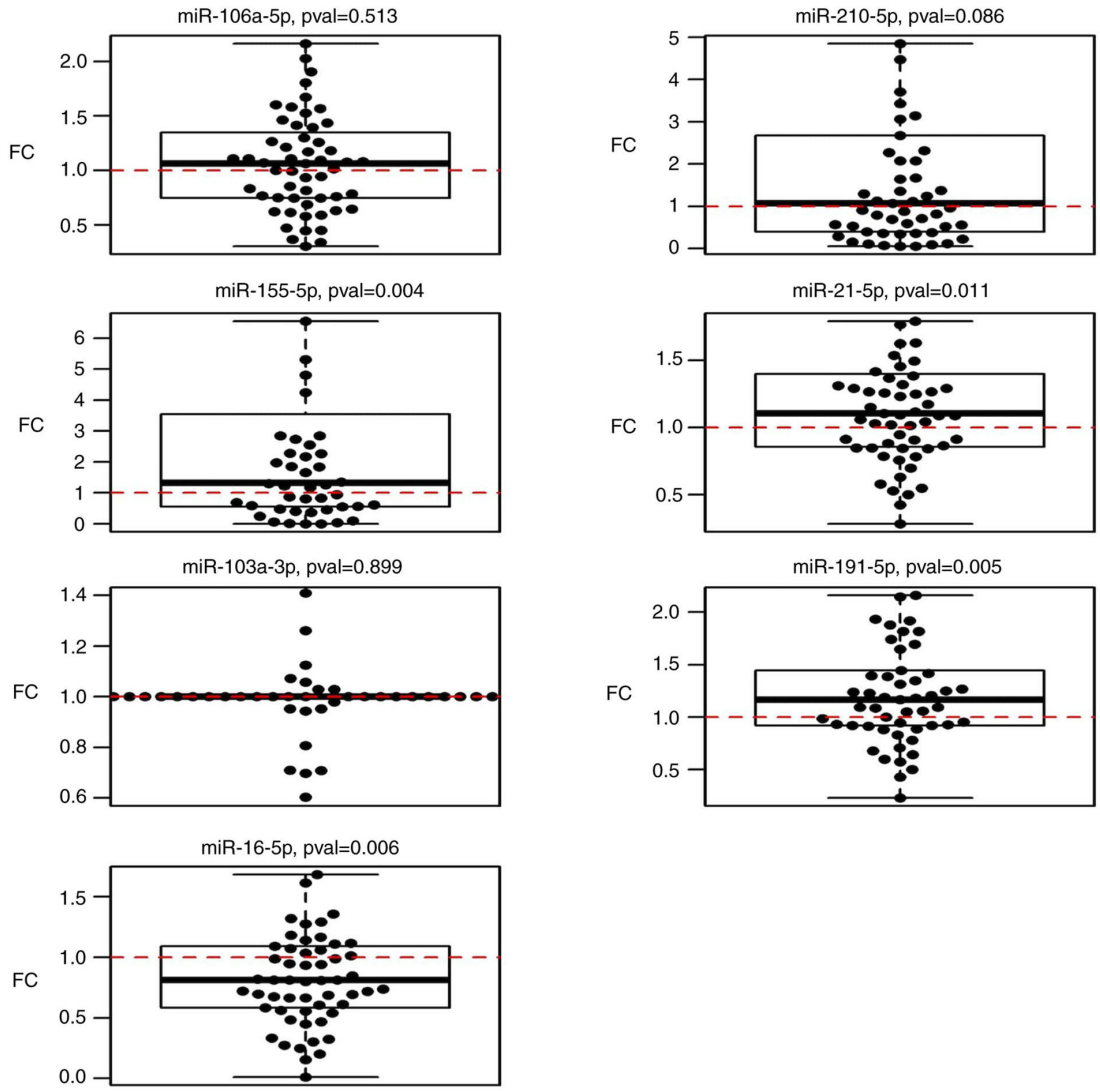

Figure 2. Boxplots displaying circulating miRNA expression in post-operative samples compared to pre-operative expression. FC, fold change; miR, microRNA; pval, P-value.

higher plasma (6,33) and/or serum (34) miR-106a expression in patients with CRC than in healthy individuals and lower miR-106 levels in post-operative samples (35). Li *et al* (34) also indicated that high miR-106a expression in patients with CRC treated with adjuvant chemotherapy is associated with poor therapeutic outcome and shorter disease-free survival (DFS).

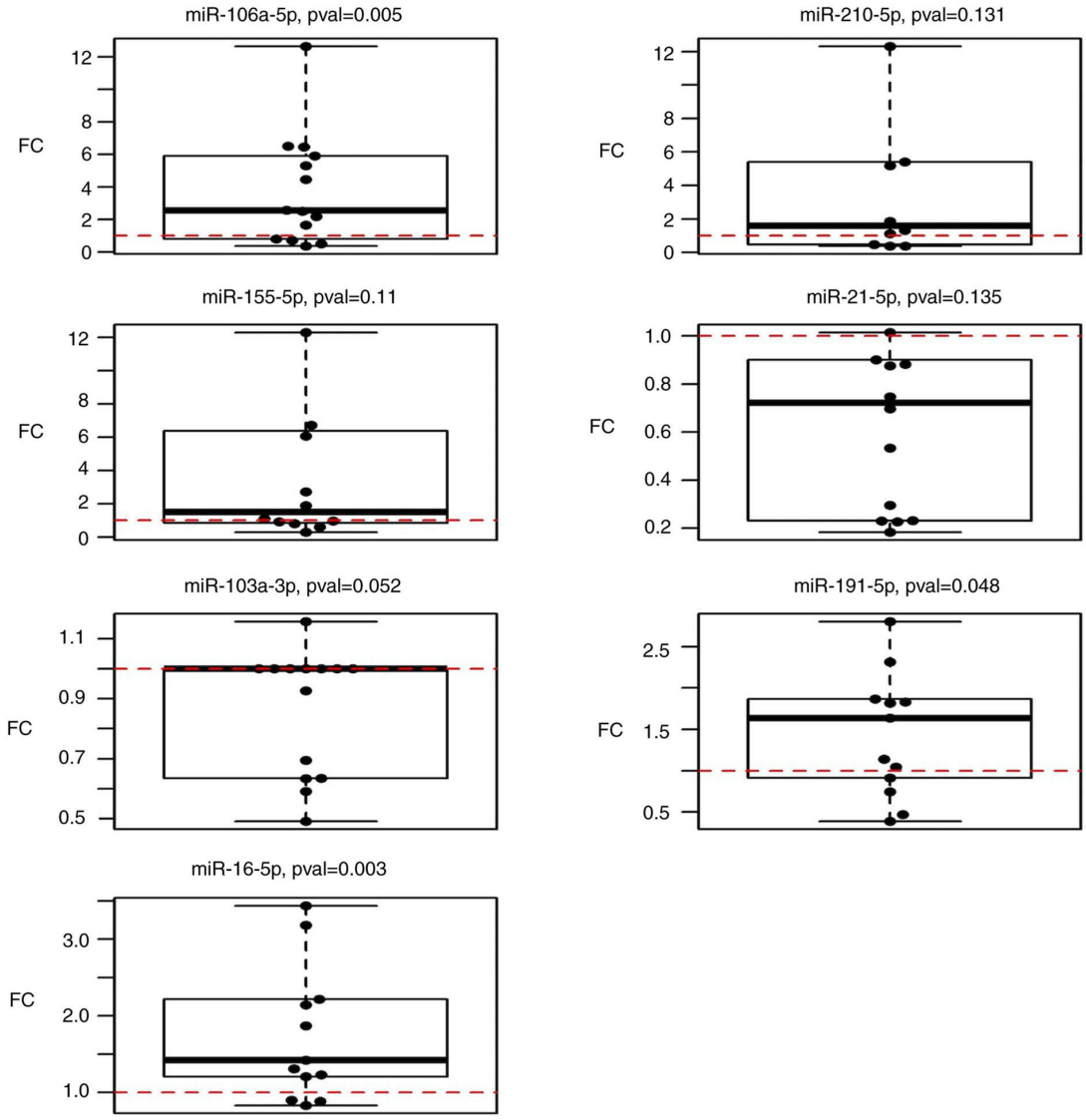

Figure 3. Boxplots displaying the expression of selected circulating miRNAs in follow-up samples (3 months after surgery) compared to the pre-operative state. FC, fold change; miR, microRNA; pval, P-value.

In addition, increased levels of miR-106a have been detected in CRC tissue compared to normal adjacent tissue, and down-regulation of miR-106a in CRC tissue has been reported as a negative prognostic marker in patients with CRC (36). On the other hand, in one study, miR-106a levels in plasma of patients with CRC exhibited no significant difference in the early post-operative period and increased 1 month after surgery (37). The results of the present study are in line with these results, although the pre-operative expression was not compared with a healthy cohort.

Studies have suggested that miR-106a is involved in the MAPK signalling pathway, focal adhesion, the FoxO signalling pathway, CRC and other malignancies (38). miR-106a has been demonstrated to be upregulated in metastatic CRC tissue and to be associated with advanced TNM stage and lymph node metastasis. Furthermore, miR-106a directly targets DLC1 and thus promotes CRC cell migration and invasion through deregulation of the Wnt/β-catenin signalling pathway (39). Upregulation of miR-106a-5p in plasma of follow-up samples may be associated with cell migration and proliferation during intestinal healing that is accompanied by similar signalling pathways as cancer.

As mentioned in the Results, the expression level of miR-155 and miR-210 in the present study was difficult to evaluate due to higher Cq values (>35 Cq) and a high standard deviation from triplicate experiments. A slightly higher expression level was detected in post-operative samples and no change in follow-up samples. High expression of miR-155 and miR-210 has been reported in CRC tissue compared to normal adjacent tissue (40-42). The serum levels of miR-155 and -210 decreased significantly 3 months after surgery and chemotherapy and were elevated again at 12-18 months after treatment and prior to the diagnosis of distant metastasis and recurrence (5,26).

Sabry *et al* (43) found that upregulation of miR-210 is associated with large tumour size, positive lymph node metastasis and local invasion. Qu *et al* (42) also suggested that upregulation of miR-210 is induced by hypoxia, which was confirmed by another study (44). Concerning the expression of miR-210, the present results did not reveal any statistically

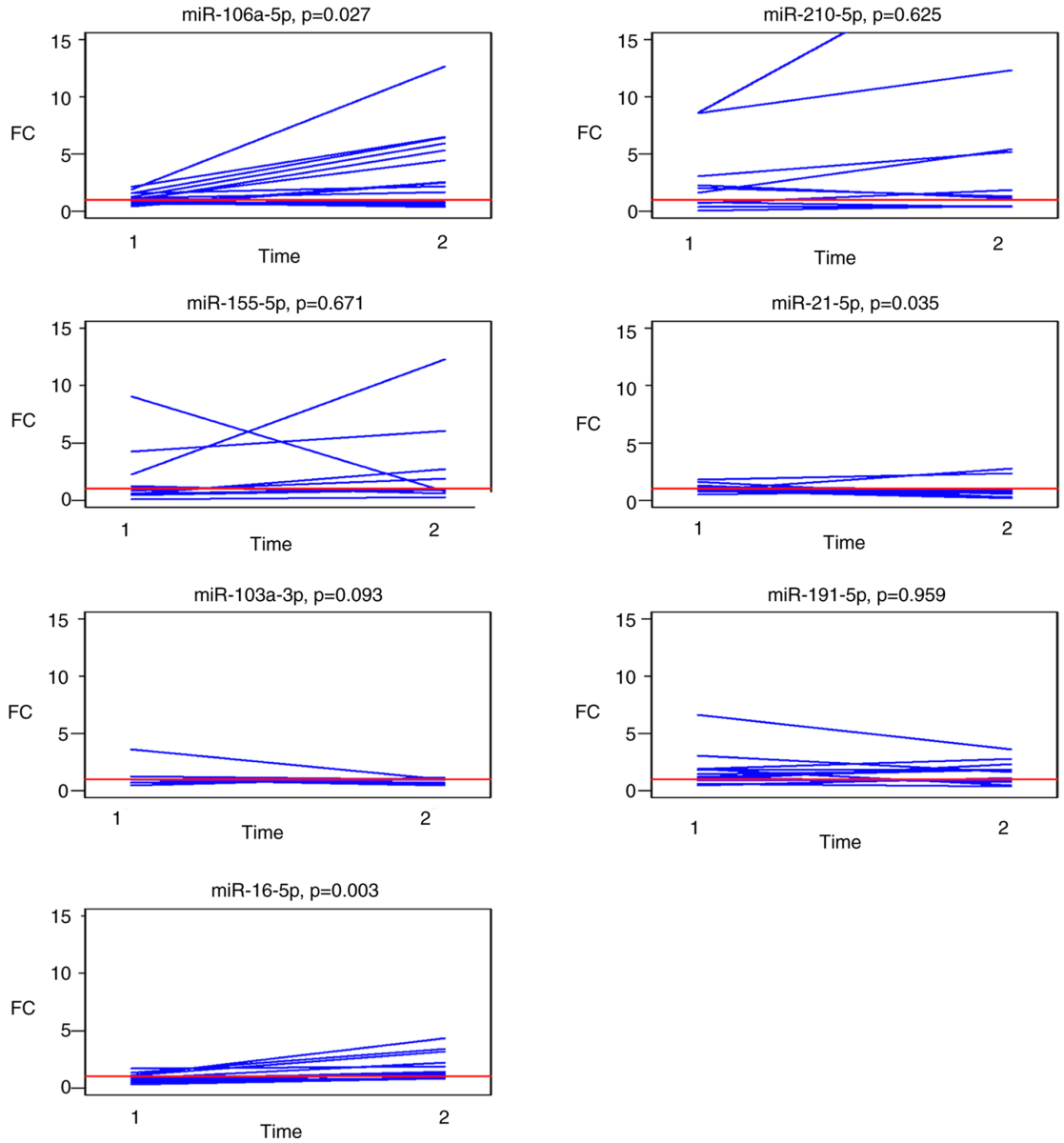

Figure 4. FCs in the circulating miRNA expression levels in paired samples from 14 patients: Post-operative samples (time-point 1) and in follow-up samples collected 3 months after surgery (time-point 2; indicated on the X-axis). Paired values are connected by lines. FC, fold change; miR, microRNA.

significant changes; however, Sabry *et al* (43) determined higher expression of miR-210 in patients with CRC and adenomas. Furthermore, the present study indicated higher miR-210 expression in patients who underwent neoadjuvant treatment. Jung *et al* (45) reported that patients with breast cancer treated with neoadjuvant chemotherapy had elevated circulating miR-210-5p and suggested that miR-210 may be used to predict the outcome and monitor therapeutic response.

miR-155 has an important role in the immune system due to targeting ~140 genes encoding immunomodulatory proteins, inflammation-related proteins and tumour suppressor proteins (46). miR-155 has been indicated to be upregulated in numerous malignancies as well as CRC (47) where chronic inflammation has an important role (48). Furthermore, miR-155 downregulates core MMR proteins and induces microsatellite instability (MSI) in CRC. Therefore, upregulation of miR-155 may have a role in tumorigenesis by combining MSI and inflammatory stimuli (49). Ulivi *et al* (50) analysed a panel of circulating miRNAs in relation to the outcome in patients with metastatic CRC treated with bevacizumab. They determined that miR-155 upregulation one month after bevacizumab treatment was associated with significantly shorter progression-free survival and overall survival (OS), and thus, miR-155 may be utilized in drug response monitoring (50).

In the present study, significant upregulation of miR-21 in post-operative samples compared to the pre-operative state and significant downregulation in follow-up samples compared to paired post-operative samples was observed. Jin *et al* (8) reported upregulation of miR-21 in pre-operative samples, post-operative miR-21 downregulation and no difference in patients with recurrence. miR-21 is an important molecule in epithelial-mesenchymal transition (EMT) and its low expression is associated with increased OS. Equivalently, higher serum miR-21 expression in patients with CRC was reported to be associated with liver metastasis and CRC recurrence (12), as well as poorer DFS and OS (24). miR-21

Table IV. Differences in the post- and follow-up vs. pre-operative levels of miRNAs from the selected panel (fold changes).

miRNA	Post-operative samples (n=14)	Follow-up samples (n=14)	P-value
miR-106a-5p	1.09 (0.78, 1.50)	2.54 (1.01, 5.77)	0.027
miR-210-5p	1.5 (0.7, 2.3)	1.6 (0.6, 5.4)	0.600
Unknown	0	4	
miR-155-5p	1.2 (0.6, 3.1)	1.5 (0.9, 6.2)	0.700
Unknown	2	2	
miR-21-5p	1.02 (0.85, 1.22)	0.72 (0.25, 0.90)	0.035
miR-103a-3p	1.00 (1.00, 1.00)	1.00 (0.65, 1.00)	0.093
miR-191-5p	1.21 (0.93, 1.92)	1.64 (0.91, 1.87)	>0.900
Unknown	1	1	
miR-16-5p	0.81 (0.63, 1.10)	1.42 (1.21, 2.22)	0.003
Unknown	2	1	

Values are expressed as the median (lower quartile, upper quartile). Wilcoxon's rank-sum test was performed for statistical comparisons. miRNA/miR, microRNA.

Table V. KEGG analysis of the significantly deregulated microRNAs in patients' plasma three months after surgery with related target genes.

KEGG pathway	Pathway ID	P-value	Targeted genes
Proteoglycans in cancer	hsa05205	6.6041×10^{-7}	STAT3, PDCD4, FRS2, IGF1R, CCND1, CTNNB1, TIMP3, VEGFA, MAPK1
Hippo signaling pathway	hsa04390	8.3834×10^{-5}	YAP1, CCND2, CCND1, CTNNB1, LATS1
Focal adhesion	hsa04510	0.0076	ITGB8, PAK2, CCND2, IGF1R, ARHGAP35, CCND1, CTNNB1, VEGFA, MAPK1, TLN1, COL4A1
Signaling pathways regulating pluripotency of stem cells	hsa04550	0.0096	STAT3, REST, IGF1R, ZFHX3, CTNNB1, SKIL, MAPK1
Prolactin signaling pathway	hsa04917	0.0096	STAT3, CCND2, SOCS6, CCND1, MAPK1
Endocrine and other factor-regulated calcium reabsorption	hsa04961	0.0096	CLTC, RAB11A
Pathways in cancer	hsa05200	0.0096	STAT3, IGF1R, APPL1, CCND1, CTNNB1, VEGFA, MAPK1, COL4A1
Colorectal cancer	hsa05210	0.0096	APPL1, CCND1, CTNNB1, MAPK1
FoxO signaling pathway	hsa04068	0.0167	STAT3, CCND2, IGF1R, CCND1, PRKAB2, SOD2, MAPK1, CCNG2
Endometrial cancer	hsa05213	0.0167	CCND1, CTNNB1, MAPK1
Thyroid cancer	hsa05216	0.027	CCND1, CTNNB1, MAPK1
Lysine degradation	hsa00310	0.0424	WHSC1, KMT2C
p53 signaling pathway	hsa04115	0.0424	CCND2, CCND1, SESN1, TNFRSF10B, CCNG2

KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, *Homo sapiens*.

expression in CRC tumour tissue was indicated to be elevated compared with that in normal adjacent tissue and miR-21 induces proliferation in CRC cell lines (51). Upregulation of miR-21 was also reported to be associated with a higher TNM stage and BRAF mutation (52). miR-21 promotes carcinogenesis by inhibiting negative regulation of the RAS/MEK/ERK pathway and also downregulates the expression of PTEN, TPM1 and PDCD4, promoting tumour progression (53). The present study indicated a slightly increased level of miR-21-5p expression immediately after surgery but a significant reduction in paired samples taken 3 months after surgery. In a study with a similar in patients with oesophageal squamous cell carcinoma, plasma expression of miR-21 was significantly reduced in post-operative samples taken 1 month after oesophagectomy (54). Furthermore, upregulation of miR-21 in post-operative samples may reflect an increased inflammatory response and oxidative stress with an increase in the oxidative stress response protein aldose reductase (AR) and

Table VI. GO annotation results of the target genes of deregulated microRNAs in subcategories biological process (top 10 GO terms were included), cellular component and molecular function.

A, Biological process

GO ID	GO term name	P-value	Target gene count
GO:0000278	Mitotic cell cycle	$<1x10^{-325}$	146
GO:0006461	Protein complex assembly	$<1x10^{-325}$	239
GO:0006464	Cellular protein modification process	$<1x10^{-325}$	744
GO:0006950	Response to stress	$<1x10^{-325}$	625
GO:0007596	Blood coagulation	$<1x10^{-325}$	150
GO:0008150	Biological_process	$<1x10^{-325}$	3807
GO:0008219	Cell death	$<1x10^{-325}$	305
GO:0009056	Catabolic process	$<1x10^{-325}$	605
GO:0009058	Biosynthetic process	$<1x10^{-325}$	1079
GO:0010467	Gene expression	$<1x10^{-325}$	286

B, Cellular component

GO ID	GO term name	P-value	Target gene count
GO:0005575	Cellular_component	$<1x10^{-325}$	3927
GO:0005654	Nucleoplasm	$<1x10^{-325}$	422
GO:0005829	Cytosol	$<1x10^{-325}$	880
GO:0043226	Organelle	$<1x10^{-325}$	2831
GO:0043234	Protein complex	$<1x10^{-325}$	1092
GO:0005815	Microtubule organizing center	$3.363976x10^{-14}$	140

C, Molecular function

GO ID	GO term name	P-value	Target gene count
GO:0000988	Protein binding transcription factor activity	$<1x10^{-325}$	165
GO:0001071	Nucleic acid binding transcription factor activity	$<1x10^{-325}$	240
GO:0003674	Molecular_function	$<1x10^{-325}$	3916
GO:0003723	RNA binding	$<1x10^{-325}$	641
GO:0008092	Cytoskeletal protein binding	$<1x10^{-325}$	244
GO:0019899	Enzyme binding	$<1x10^{-325}$	475
GO:0030234	Enzyme regulator activity	$<1x10^{-325}$	242
GO:0043167	Ion binding	$<1x10^{-325}$	1563
GO:0044822	Poly(A) RNA binding	$<1x10^{-325}$	532
GO:0032182	Small conjugating protein binding	$1.622062x10^{-9}$	40

GO, Gene Ontology.

activation of PTEN-induced apoptosis and controlled cell growth. Repression of AR led to inhibition of colon cancer cell growth by downregulation of miR-21 expression and upregulation of PTEN and FOXO3a expression (hsa04068). However, the specific mechanism was not elucidated (55).

Hong *et al* (56) detected upregulation of miR-103 in CRC cell lines. Zheng *et al* (57) determined upregulation of miR-103 in CRC tissue compared to normal adjacent tissue. Furthermore, they demonstrated that patients with high miR-103 expression had poorer OS. Wang *et al* (58) reported upregulation of miR-103a-3p in serum of patients with CRC and in those with recurrence. There is currently a lack of studies evaluating the role of miR-103a-3p in carcinogenesis due to its use as a housekeeping gene as discussed below.

Another significantly upregulated miRNA in post-operative samples of the present study was miR-191-5p. There was also a weak significant association between the level of miR-191 and tumour stage and microsatellite-stable cancers. The function of miR-191 in CRC remains to be fully elucidated. However,

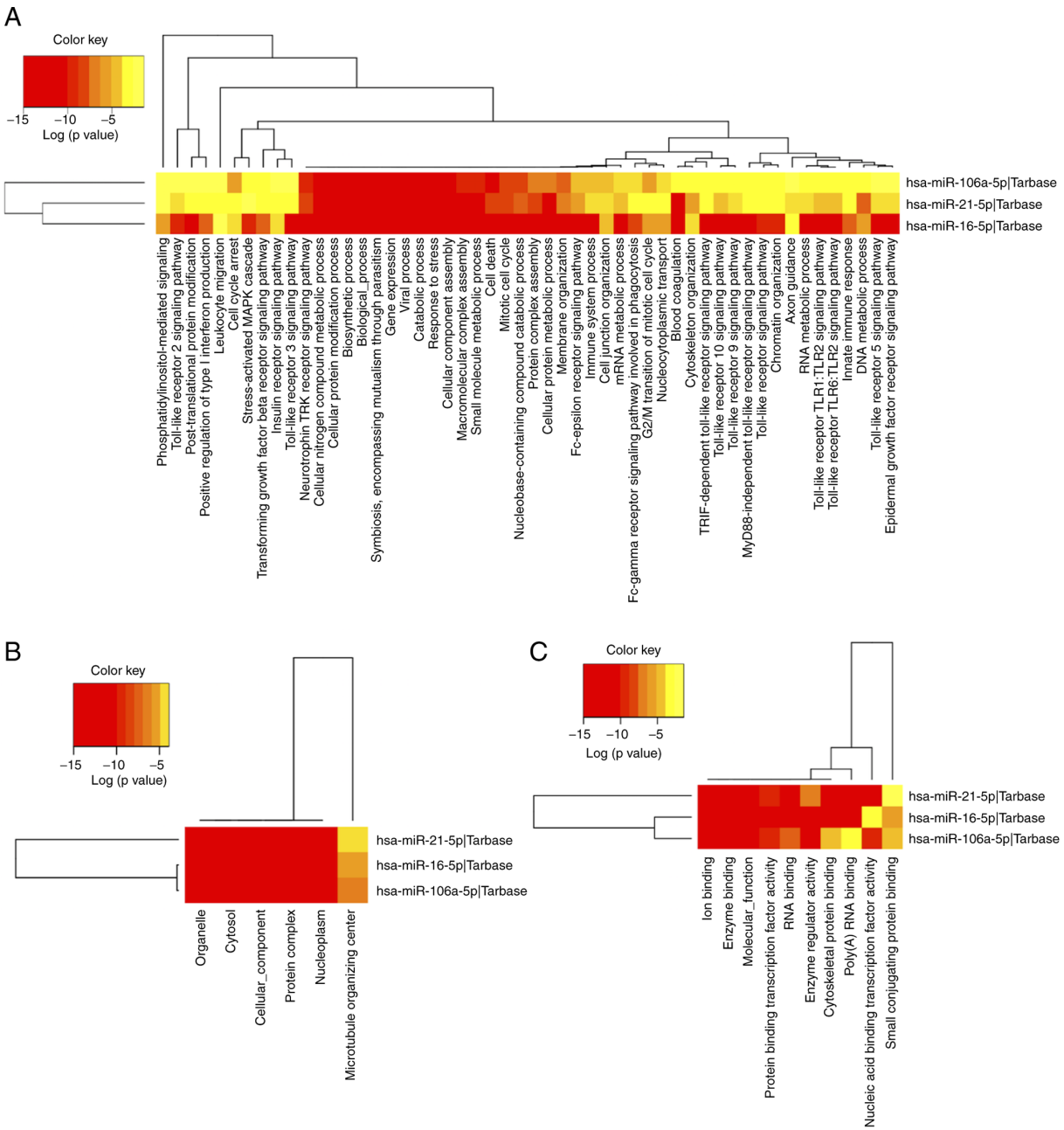

Figure 5. Heatmaps of GO analysis. GO enrichment of supposed targets was performed for three significantly deregulated miRNAs at three subcategories: (A) Biological process, (B) cellular component and (C) molecular function. Diverse levels of enrichment of pathway terms in the miRNA target genes are displayed by different colors, the red represents a higher degree of enrichment. GO, Gene Ontology; FC, fold change; miR, microRNA, *hsa, Homo sapiens*.

several studies have reported deregulation of miR-191 in various cancer types and diseases. It has been indicated that miR-191 overexpression induces progression of hepatocellular carcinoma and intrahepatic cholangiocarcinoma and promotes EMT in metastatic bronchial epithelial cells (59). Zhang *et al* (60) reported that upregulation of miR-191 promoted proliferation in CRC and reduced cell susceptibility to 5-fluorouracil. Qin *et al* (61) demonstrated that high expression of miR-191 is significantly associated with advanced TNM stage, liver metastasis and unfavorable prognosis in patients with CRC. On the other hand, Milanesi *et al* (62) and Chen *et al* (63) revealed that downregulation of miR-191 in patients with KRAS-mutation CRC and low miR-191 expression is correlated with poor prognosis.

In the present study, the level of miR-16-5p expression in patients' plasma after surgery was significantly decreased (P=0.006) compared to the pre-operative state and elevated in paired follow-up samples (P=0.003). Previous studies have demonstrated a tumour-suppressive role of miR-16 in the progression of several malignancies (64), including CRC (65). It has been indicated that overexpression of miR-16 inhibited proliferation and induced apoptosis by regulation of the p53/survivin signalling pathway (66). Several studies have revealed downregulation of miR-16 in malignant CRC tissue when compared to adjacent tissue and identified an association between low miR-16 expression and histological parameters such as advanced TNM stage and poor histological grade, as well as a higher incidence of lymph node metastases and

tumour recurrence. Low miR-16 expression is correlated with shorter DFS and OS (64,65,67). Ostenfeld *et al* (68) analysed miRNA profiles in epithelial-derived extracellular vesicles (EVs) secreted by cancer cells and observed a significantly reduced level of miR-16-5p post-operatively.

miR-16-5p, miR-103a-3p and miR-191-5p are commonly used for qPCR data normalization, which is a critical step in each gene expression experiment. Danese *et al* (25) aimed to identify the ideal reference miRNAs in CRC from miRTarBase and came to the conclusion that miR-16 and -103 target onco/tumour suppressor genes are not suitable for normalization. The expression of miR-16-5p in blood plasma is influenced by a varying level of haemolysis (29). Therefore, in the present study, samples without haemolysis were carefully selected despite the overall reduction in the number of samples in the test groups. Proper data normalization is the most important part of each study and may influence its overall results. In studies dealing with circulating miRNA expression, the issue prevails that there are no suitable housekeeping miRNAs expressed equally across the various physiological conditions of patients. Other procedures advise normalization to synthetic spike-ins added through the process of sample preparation prior to analysis. However, the use of synthetic spike-ins does not correspond to the physiological and biological conditions under investigation and they do not have the same effect as the housekeeping genes. Another obstacle was not having the same input of RNA/miRNA concentration across the samples collected from the patients at various time-points. Therefore, median normalization was used, which is more frequently used in next-generation sequencing (69,70) and microarray (71) data normalization.

Some oncomiRs were found to be downregulated 1 week after surgical removal of the tumor (17,20). It may be speculated that upregulation of oncomiRs in post-operative samples may be associated with post-operative wound healing. The present study demonstrated a decrease in these oncomiRs later at a follow-up sampling. At present, intestinal wound healing is only partially understood. It is predominantly studied in inflammatory bowel disease as well as in Crohn's disease and ulcerative colitis. During acute and chronic intestinal inflammation, immune cells such as neutrophils and macrophages induce local tissue damage by secreting tissue-degrading enzymes, reactive oxygen radicals and pro-inflammatory cytokines. The process promotes migration of myofibroblast cells to the site of the defect, arranging contractility of the wound area and the production of extracellular matrix (ECM) (72). ECM is composed of macromolecules such as proteoglycans (pathway hsa05205), non-proteoglycan polysaccharides, proteins such as collagen and elastin, and EVs. ECM also has a major function in cell adhesion (hsa04510) (73) and intestinal healing is also associated with intestinal stem cell differentiation and proliferation (hsa04550) (74,75). Notably, Hippo signalling (hsa04390) also has a role in regulating the regeneration of organs such as the liver, heart, nervous system and skin as well as intestine (76) and also prolactin (hsa04917) has been suggested to have an important role in re-epithelialization and promote wound healing (77). According to a previous study, plasma and wound fluid levels of proangiogenic proteins were elevated in patients after CRC resection for 3 to 5 weeks, leading to the hypothesis that the wound healing may stimulate tumour growth in residual tumour deposits (78).

The tumour microenvironment (TME) is composed of blood vessels, ECM, RNA, secreted proteins, small organelles and various populations of stromal cells (fibroblasts, adipocytes, pericytes and immune/inflammatory cells) (79). TME cells have a role in the communication between them, which is performed in autocrine, paracrine and/or endocrine ways. These cells produce growth factors and cytokines which modify molecular and cellular processes and thus alter the maturation of the TME (80).

In general, the TME participates in immune cell recruitment and activation, ECM alterations and angiogenesis, which contribute to tumour progression and wound healing (81). ECM proteins are an important component of the TME and it is well-known that changes in the composition of ECM proteins are responsible for the genesis of CRC (82). Fibrous proteins, proteoglycans, collagen and elastin as the major types of ECM proteins provide structural support and elasticity in all tissues (83). Various studies suggested that expression of high levels of proinflammatory cytokines, such as TNF produced by tumour-associated macrophages, induces neoplasm growth and invasion (84). Inflammation is involved in CRC development and progression and in post-operative intestinal wound healing. Chronic inflammation is linked to alterations in the TME through cytokines, chemokines and growth factors (85). Li *et al* (86) reported that elastin recombinant protein increased the proliferation of CRC epithelial cells, induced EMT, increased TNF secretion by bone marrow-derived macrophages and reduced E-cadherin in CRC epithelial cells. Inflammation is a trigger of ECM remodelling and furthermore, Li *et al* (86) demonstrated that proteins of ECM regulate inflammation.

In the present study, the panel of selected miRNAs was investigated in plasma of patients with CRC at the pre-operative stage, in the early post-operative period and 3 months after surgery. None of the patients received any chemotherapy at the time of follow-up sampling. Significant upregulation of miR-155-5p, miR-21-5p and miR-191-5p, and downregulation of miR-16-5p was determined. In paired follow-up samples, the most relevant upregulation was observed for miR-106a-5p and miR-16-5p and downregulation for miR-21-5p. Pathway analysis outlined their role in cancer development, but the same pathways are also involved in controlled wound healing and regeneration of intestinal epithelium (85,87). Of note, the present study is limited by its sample size as well as lack of samples in the follow-up group. Furthermore, functional studies are necessary to confirm the present results in association with the pathways identified by the *in silico* analysis. It may be speculated that miRNA expression associated with intestinal wound healing influences the expression levels in patients' circulation (87) and the healing process should be considered in studies focusing on disease recurrence (45). For post-operative sampling, a longer interval after surgery (4-6 weeks) (88) may help in the early identification of patients with CRC recurrence in the case of latent metastases. However, more studies are necessary to confirm these results.

The selected miRNAs seemed to be promising markers for the monitoring of miRNA expression in the patients' circulation. However, the present results require confirmation in a larger cohort of patients. miRNA studies should also consider

all of the physiological conditions that are taking place in real time in the organism and are not influenced by sample processing and technical variations. Identifying appropriate biomarkers to determine a higher risk of CRC recurrence and biomarkers to predict patient response to adjuvant treatment is a novel way to monitor and treat patients. Based on these results, it may be possible to select patients in need of more intensive monitoring as well as patients who are unlikely to benefit from adjuvant therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization, ZL and LL; methodology, ErK, VH and ZK; software, VH, ZK, MG; validation, VH and ZK; formal analysis, VH and MG; investigation, EvK, VH, MSa, BV, PM and MSm; resources, ErK, VH, MSa, BV, PM and MSm; data curation, EvK, VH, ZK, MG and PM; writing-original draft preparation, EvK and VH; writing - review and editing, MSa, ErK, LL and ZL; supervision, ZL and LL; project administration, ZL; funding acquisition, ZL. VH and ErK confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients were informed about this study and provided written informed consent. This research was approved by the Ethical Committee at Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava (Martin, Slovakia).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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