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Original Articles

Longitudinal monitoring reveals dynamic changes in circulating tumor cells (CTCs) and CTC-associated miRNAs in response to chemotherapy in metastatic colorectal cancer patients

Karen Tan [a, **], Sai Mun Leong [c], Zizheng Kee [c], Patrick Vincent Caramat [a], James Teo [a], Michael Vito Martin Blanco [b], Evelyn S.C. Koay [c], Wai Kit Cheong [d], Thomas I-Peng Soh [b], Wei Peng Yong [b], Angela Pang [b, *]

[a] Department of Laboratory Medicine, National University Hospital, Singapore
[b] National University Cancer Institute, Singapore
[c] Department of Pathology, National University, Singapore
[d] Department of Surgery, National University Hospital, Singapore

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ABSTRACT

We evaluated the changes in CTC count and CTC-associated miRNAs during the course of chemotherapy in patients with metastatic colorectal cancer. Blood samples were collected from 9 metastatic colorectal cancer patients prior to chemotherapy and at every other chemotherapy session during the course of treatment. CTCs were isolated and enumerated using a size-exclusion method (CellSievo, Singapore). CTC-associated miRNAs were isolated using a paper-based, partitioning method, and analyzed using reverse transcription quantitative real-time PCR (MiRXES, Singapore). CTC count trends generally correlated with disease progression defined by radiological measurements and trends in carcinoembryonic antigen (CEA) levels; hence CTC counts may be useful in cases where CEA is not elevated. CTC-associated miRNAs identified were miR-15b, miR-16, miR-19a, miR-21, miR-25, miR-30d, miR-126, miR-185, miR-221, miR-222, and miR-324−5p. The expression of CTC-associated miRNAs did not appear to correlate with CTC count and exhibited inter-individual heterogeneity. This pilot study suggests that analysis of CTC changes during the course of treatment may be useful in monitoring response to therapy in metastatic colorectal cancer.

Introduction

Colorectal cancer is the third most common cancer and the fourth most common cause of cancer-related death worldwide [1]. First-line chemotherapy for metastatic colorectal cancer includes the combinations of 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI), 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX), capecitabine and oxaliplatin (XELOX), and the combination of 5-fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) [2]. The use of these combination therapies for metastatic colorectal cancer have led to response rates of >50% and median survival of up to 2 years [3,4]. However, practically all metastatic colorectal cancers eventually become resistant to chemotherapy [5]. Therefore, the development of biomarker assays to predict resistance and the identification of alternative strategies to overcome chemotherapeutic resistance are important in reducing the morbidity and mortality. Candidate predictive biomarkers for chemotherapy include mutant *TP53*, thymidylate synthase (TS) expression, amplified ERCC1, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and mutant *BRAF* [6]. However, none of these are recommended in clinical guidelines as companion diagnostics for colorectal cancer treatment [7] and

Abbreviations: CTCs, Circulating tumor cells; miRNA, microRNA; CEA, Carcinoembryonic antigen; RECIST, Response Evaluation Criteria In Solid Tumors; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; WBC, white blood cells.

* Corresponding author. Department of Haematology-Oncology, National University Hospital, 1E Kent Ridge Road, NUHS Tower Block Level 7, 119228, Singapore.
** Corresponding author. Department of Laboratory Medicine, National University Hospital, Singapore.
E-mail addresses: karen_ml_tan@nuhs.edu.sg (K. Tan), angela_pang@nuhs.com.sg (A. Pang).

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require further validation studies.

Recently, targeted biologic therapies such as bevacizumab targeting the vascular endothelial growth factor (VEGF) and cetuximab and panitumumab targeting the endothelial growth factor receptor (EGFR) have demonstrated significant survival benefits in metastatic colorectal cancer alone or in combination with cytotoxic chemotherapy [8,9]. However, patients respond differently with respect to efficacy and toxicity, therefore it is important to identify biological biomarkers that can predict response to therapy and hence identify patients that would benefit from the therapy. *KRAS* and *NRAS* mutation status are clinically useful to predict response to anti-EGFR therapy [7]. Other molecular markers such as *PIK3CA* and *BRAF* are not yet established for prediction of response to therapy [7].

Circulating tumor cells (CTCs) are cancer cells dislodged from the primary tumor or its metastases and disseminated in the blood stream [10]. CTCs can be isolated directly from peripheral blood, obviating the need for invasive tumor biopsies [11]. Many studies have shown that CTCs may be used to predict disease progression and survival in metastatic cancer [12,13]. Molecular profiles obtained from isolated CTCs can be correlated with treatment outcomes [14] and may reveal druggable candidates. Despite advances in technology that have allowed the development of assays for using CTCs as biomarkers of disease progression and therapeutic response, the kinetics of the changes in numbers and molecular characteristics of CTCs over time remain poorly understood [15]. Because of these limitations, despite much evidence for their prognostic value, CTCs have not yet been recommended clinically to guide decisions on therapy as data demonstrating the clinical utility of CTC-based tests are lacking [7,16].

MicroRNAs (miRNAs) are short (18-22-nucleotides) RNA that bind to complementary sequences in the 3′ untranslated region of multiple target messenger RNAs [17]. By either blocking translation or inducing target mRNA degradation, miRNAs regulate multiple biological processes, and are implicated in pathological processes [17]. Specific miRNA expression patterns are associated with cancer, and miRNAs have been suggested as diagnostic and predictive clinical biomarkers [18]. Few studies have examined the expression of miRNA in CTCs [13,19], and to the best of our knowledge, there is currently no published study on longitudinal CTC-specific miRNA expression in metastatic colorectal cancer.

We previously reported a paper-based method to efficiently extract miRNAs from CTCs [20]. Using this digital-PCR inspired method, background miRNA expression was excluded from contaminating blood cells, and CTC-specific miRNA expression profiles were derived [20]. In this study, we evaluated the changes in CTC count and CTC-associated miRNAs during the course of chemotherapy in patients with metastatic colorectal cancer by comparing with CEA levels and imaging results. We hypothesized that CTC count and miRNA expression changes during the course of treatment reflect response to therapy and may be used to assess the efficacy of ongoing treatment.

Materials and methods

Patients

This prospective single-institution study enrolled patients with the following inclusion criteria:

1. Histologically proven metastatic colorectal carcinoma where XELOX/mFOLFOX-6/XELIRI/FOLFIRI/XELOX + bevacizumab/ mFOLFOX-6 + bevacizumab/XELIRI + bevacizumab/ FOLFIRI + bevacizumab regimen is indicated and planned for chemotherapy;
2. Patient age ≥21 years;
3. Measurable disease according to RECIST criteria or evaluable disease;
4. A life expectancy of at least 3 months;
5. Patient signed informed consent.

A patient meeting any of the following criteria was excluded:

1. Patients who had not recovered from major surgery;
2. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, other serious uncontrolled concomitant disease, psychiatric illness/social situation that would limit study compliance, at the discretion of the investigator;
3. Known allergies to any component of the drug regime;
4. Known dihydropyrimidine dehydrogenase deficiency.

At least 10 mL of peripheral blood samples were collected in spray-coated K2-EDTA vacutainer tubes from the patients at baseline, before the start of the first chemotherapy session, and before every other chemotherapy session, over the course of chemotherapy, until clinical or radiological determination of disease progression as determined by the attending oncologist or one year's duration, whichever was earlier. Evaluation of the clinical response was performed by computed tomography according to RECIST version 1.1 criteria [21]. Information about the number and sites of metastases, carcinoembryonic antigen (CEA) levels in serum, tumor genetics (*KRAS* mutation status of the tumor); clinical, radiological and pathological data were gathered from the clinical records. The study protocol was approved by the local ethics review board (NHG DSRB 2014/00713).

CTC detection

The blood was diluted 1:1 with PBS/5 g/L BSA/2 mM EDTA and the diluted blood was filtered using a 40 μm cell strainer (Becton Dickinson, USA) directly into the funnel filtration system and filtered through the microsieve (CellSievo, Singapore) at a flow rate of 500 μL of blood per minute (min), as described previously [22]. Four 1-mL washes with PBS/5 g/L BSA/2 mM EDTA were performed, followed by a 30-min incubation with a 100-μL antibody mixture containing 20 μL 100 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA), 20 μL AlexaFluor-488 conjugated anti-CD45 antibody (catalogue number: MCA87A647, AbD Serotec, UK), 20 μL PE-conjugated anti-EpCAM (catalogue number: 324206, BioLegend, USA), and 20 μL PBS buffer. This was followed by four 1-mL washes with EDTA-free PBS/5 g/L BSA. CTCs were eluted in 500 μL PBS/5 g/L BSA from the microsieve by reversing the flow of the pump and collected in sterile 1.5-mL tubes (Eppendorf, Germany). The eluate containing a mixture of RBCs, WBCs and CTCs was centrifuged at 2000 rpm for 5 min. Most of the supernatant was removed. The cell pellet was resuspended in 5 μL PBS/5 g/L BSA. The eluate was spotted onto the wells of a MicroWell™ Minitray (catalogue number: 452256; Nunc, Denmark) with 0.5 μL in each well. Each well was examined under a BX61 fluorescence microscope (Olympus, Japan). WBCs fluoresced blue (DAPI) and green (AlexaFluor-488 conjugated anti/CD45). CTCs fluoresced blue (DAPI) and occasionally red (EpCAM) but not green. Using this method, we have previously shown that CTC counts are significantly higher in breast and colorectal cancer patients but not healthy controls [20].

miRNA extraction

CTC-containing wells were pooled together for miRNA

extraction using the Flinders Technology Associates (FTA®) Elute card (GE Healthcare Life Sciences, UK), along with an equal number of CTC-null wells to serve as background control, as described previously [20]. A small piece of the FTA® Elute card (5 mm × 5 mm) was pressed against the bottom of the microwell with moderate force for 5 s (s) to soak up and lyse the cells. The discs were transferred to fresh 1.5-mL Eppendorf tubes (Germany) and washed in 500 μL 70% ethanol for 5 min before drying. The dried discs were soaked in 12–20 μL of diethyl pyrocarbonate (DEPC)-treated water. miRNAs were eluted at 95 °C for 30 min. During the process, different forceps were used to handle the CTC-containing and CTC-null samples respectively to avoid cross-contamination.

CTC-associated miRNA analysis by RT-qPCR

Twenty-four miRNAs were analyzed in the CTC samples. These were selected based on miRNA expression profiling in oxaliplatin-resistant versus oxaliplatin-sensitive colorectal cancer cells [21] and literature search for their roles in colorectal cancer (Table 1). Reverse transcription (RT) was performed using stem-loop primers (MiRXES, Singapore) [20,22] in three multiplexed reactions (group A: miR-16, miR-21, miR-34, miR-93, miR-106a, miR-200c; group B: miR-221, miR-222; group C: miR-15b, miR-19a, miR-25, miR-30a, miR-30d, miR-100, miR-125b, miR-126, miR-185, miR-192, miR-196b, miR-202, miR-215, miR-324–5p, miR-744, miR-601). 2 μL of total miRNAs extracted from the FTA® elute card was used along with 100 nM of each primer for each reaction in a total volume of 10 μL. The RT reaction was conducted at 42 °C for 30 min, followed by 95 °C for 5 min using a thermal cycler (Eppendorf, Germany). Real-time qPCR was performed on the 7900HT real-time PCR instrument (Thermo Fisher Scientific, USA) using IDEAL miRNA assays (MiRXES, Singapore). 2.5 μL of each 1:10 diluted cDNA sample was subjected to qPCR in a total volume of 10 μL in 5X IDEAL miRNA qPCR Master Mix with 1X miRNA-specific qPCR assays (MiRXES, Singapore). Thermocycling of cDNAs was performed with 10 min of initial denaturation at 95 °C and 5 min at 40 °C, followed by 40 cycles of 10 s denaturation at 95 °C and 30 s annealing/extension at 60 °C.

Each cDNA sample was run in duplicates for the qPCR stage. Raw threshold cycle (C_q) values were calculated using the 7500 software v2.0.5 (Thermo Fisher Scientific, USA) with automatic baseline and threshold settings. Only C_q values below 35 were used, without modification; Cq values that were above 35 were taken as 35 for calculation. The difference in C_q values was calculated for the two fractions ($\Delta C_q = C_q$ of CTC-containing wells - C_q of CTC-null wells). Normalization was performed by calculating the mean ΔC_q of all miRNAs detected for each sample at each time-point and normalizing all miRNAs to the mean ΔC_q ($\Delta\Delta C_q$). Fold difference was obtained ($2^{-\Delta\Delta Cq}$) and this value indicates the extent of miRNA expression due to CTCs only. Using this method, we have previously shown that CTC-associated miRNA expressions are significantly higher in breast and colorectal cancer patients but not in healthy controls [20]. A threshold for miRNA detection was set at the fold difference of 2.30 (corresponding to $\Delta Cq = -1.2$), as previously determined [20].

Results

Clinical characteristics of patients

All patients were diagnosed with colorectal cancer at clinical stage IV with distant metastases and were treated with oxaliplatin-based chemotherapy. A summary of the clinical characteristics of the patients is shown in Table 2. Three patients had partial response, four patients had stable disease and two patients had progressive disease as determined by Response Evaluation Criteria In Solid Tumors (RECIST) criteria [23].

Longitudinal monitoring of CTCs

We measured CTC counts and CTC-associated miRNA expression in patients during successive cycles of chemotherapy at every other cycle. We compared CTC counts with CEA levels and patient response to chemotherapy based on RECIST criteria. The changes in CTC counts reflected the disease progression and/or response to chemotherapy and correlated well with changes in CEA levels (Fig. 1). For case 1, the CTC counts remained stable while CT imaging of the liver and lung metastases showed stable disease, then increased significantly before the CT imaging showed progressive disease (Fig. 1). In case 2, the CTC counts decreased as CT imaging showed partial response of the liver metastases and colonic mass, then increased before the CT imaging showed progressive disease (Fig. 1). Similarly for case 3, the CTC counts decreased as the CT imaging of the liver metastases showed partial response, then remained relatively stable while the CT imaging showed stable disease (Fig. 1). In cases 4, 6, 7 and 8, CTC counts decreased as CT imaging showed decrease in size of metastases (partial response) (Fig. 1). For cases 5 and 9, CTC counts were relatively stable throughout the course of chemotherapy, while CT imaging showed stable disease (Fig. 1). On the whole, the trend of CTC counts increased together with CEA at times when there was progressive disease (PD) clinically (cases 1, 2) and decreased when there was partial response (PR) clinically (cases 2, 3, 6) and remained relatively stable when there was stable disease (SD) clinically (cases 1, 3, 5, 9). These results suggest that trending CTC counts during the course of chemotherapy in colorectal cancer may be useful to monitor patient response and disease progression, especially in cases where CEA levels may be uninformative (cases 4, 7 and 8).

CTC-associated miRNA expression

The expression of all 24 miRNAs screened in all 9 patients at all time-points is shown in Fig. 2. CTC-associated miRNAs that were expressed in majority of the patients across multiple time-points include miR-15b, miR-16, miR-19a, miR-21, miR-25, miR-30d, miR-126, miR-185, miR-221, miR-222, and miR-324–5p (Fig. 2). For most patients, the expression of CTC-associated miRNAs appeared transient and fluctuating during the course of chemotherapy (Fig. 2). High levels of CTC-associated miRNA expression could be observed when CTC counts were low, e.g., case 3, after 4th and 8th cycle (Figs. 1 and 2), suggesting that few CTCs may have very high levels of miRNA expressed.

Baseline CTC-associated miRNA expression did not appear to correlate with initial tumor volume of metastases, baseline CTC levels, or baseline CEA levels. The expression of CTC-associated miRNAs longitudinally over the course of chemotherapy did not appear to correlate with CTC count and exhibited inter-individual heterogeneity.

Discussion

CTCs have been represented as “liquid biopsies” of solid tumors and may potentially be used as biomarkers to assess the patient's disease progression and response to therapy [10,11]. In order to validate CTCs as biomarkers of prognostic significance, we investigated how CTC counts and CTC-associated miRNAs correlate with established clinical parameters over the course of chemotherapy in metastatic colorectal cancer patients. In this study we observed that the changes in CTC counts reflected the disease progression

Table 1
Panel of 24 miRNAs analyzed and their roles in colorectal cancer.

miRNA	Role(s) in colorectal cancer	References
miR-15b	Increased plasma miR-15b in metastatic CRC patients; Predicts metastasis in colorectal cancer	[34] [35];
miR-16	Predicts relapse and poor survival in colorectal cancer	[36]
miR-19a	Promotes colorectal cancer proliferation; Associated with lymph node metastasis; Predicts resistance to FOLFOX chemotherapy	[31] [32] [37];
miR-21	Potential prognostic marker in colorectal cancer; Increased expression in colorectal cancer	[30] [38] [67];
miR-25	Upregulated in oxaliplatin-resistant HCT-116 cells; Increased expression in colorectal adenocarcinomas and metastases	[21] [29];
miR-30a	Downregulated in colorectal cancer	[39] [40];
miR-30d	Increased in 5-fluorouracil (5-FU) resistant colorectal cancer cells	[41]
miR-34	Inhibits epithelial-to-mesenchymal transition (EMT); Downregulated in colorectal cancer	[42] [43];
miR-93	Downregulated in oxaliplatin-resistant HCT-8 cells; Downregulation predicts poor prognosis in colorectal cancer; Increased expression in colorectal cancer	[44] [45] [46];
miR-100	Downregulation predicts poor prognosis	[47]
miR-106a	Initiates migration and invasion in colorectal cancer; Suggested fecal biomarker for colorectal cancer	[48] [49];
miR-125b	Prognostic marker in colorectal cancer; Confers resistance to 5-FU; Promotes metastasis	[50] [51] [52];
miR-126	Downregulation predicts poor prognosis	[53]
miR-185	High expression associates with poor survival and metastasis; Inhibits proliferation of colorectal cancer cells	[54] [55];
miR-192	Reduced expression in metastatic colorectal cancer cells; Downregulation predicts poor survival	[56] [57];
miR-196b	High expression correlates with poor prognosis	[58] [59] [60];
miR-200c	High expression in metastatic colorectal cancer	[60] [61] [62];
miR-202	Upregulated in oxaliplatin-resistant HCT-116 cells; May predict response to neoadjuvant chemotherapy	[21]] [63];
miR-215	Downregulation predicts poor survival; High levels predict poor survival	[57] [64];
miR-221	Promotes colorectal cancer metastasis; Increased expression in colorectal cancer; Suggested fecal biomarker for colorectal cancer	[65] [66] [67];
miR-222	Upregulated in oxaliplatin-resistant HCT-116 cells; Downregulated in 5-FU resistant HCT-8 and HCT-116 cells	[21] [44];
miR-324-5p	Upregulated in oxaliplatin-resistant HCT-116 cells	[21]
miR-601	Upregulated in oxaliplatin-resistant HCT-116 cells; Decreased plasma miR-601 in colorectal cancer	[21] [68];
miR-744	Upregulated in oxaliplatin-resistant HCT-116 cells	[21]

Table 2
Clinical characteristics of metastatic colorectal cancer patients. Baseline CTC and CEA values were obtained before the start of chemotherapy.

Case	Gender	Age	Primary Tumor	Metastases (organs)	Total initial metastases volume (mm)	Baseline CEA (μg/L)*	Baseline CTC (count/ 7.5 ml blood)*	KRAS	Chemotherapy	Response
1	Male	69	Rectosigmoid colon	Lungs, liver	103	960	12	MUT	XELOX	Progressive disease
2	Female	65	Ascending colon	Lungs, liver	230	216.8	30	MUT	XELOX	Progressive disease
3	Female	55	Sigmoid colon	Liver	113	221.6	33	WT	FOLFOX + panitumumab	Stable disease
4	Female	30	Rectum	Lungs	16	0.9	49	WT	XELOX	Partial response
5	Male	71	Sigmoid colon	Lungs, liver, brain	88.6	24.7	26	MUT	FOLFOX	Stable disease
6	Female	62	Sigmoid colon	Liver	82	90.1	29	WT	FOLFOX + bevacizumab	Partial response
7	Male	73	Caecum	Lungs, liver	13	3.6	16	MUT	FOLFOX	Stable disease
8	Male	58	Rectum	Lungs, liver	66	17.2	41	MUT	FOLFOX + bevacizumab	Partial response
9	Male	54	Sigmoid colon	Peritoneum	40	136.9	14	WT	FOLFOX + cetuximab	Stable disease

and/or response to chemotherapy and correlated well with changes in CEA levels (Fig. 1). This suggests that CTC monitoring may be useful to monitor the disease progression and response to chemotherapy in metastatic colorectal cancer patients, together with CEA levels. In this study we used a sieve method that allows us to obtain viable cells for miRNA isolation [20,24]. CTCs found in the circulation may have undergone epidermal-to-mesenchymal transition (EMT), a process in which CTCs lose their epithelial markers such as CK and EpCAM [11,25]; and EMT has been suggested to play a role in the metastatic process performed by CTCs [25]. Therefore we included EpCAM-negative CTCs in the CTC counts in order to capture CTCs that have undergone EMT and CD45-negative, DAPI-positive cells regardless of EpCAM status were all considered as CTCs [20]. It would be of interest to determine whether CTCs enumerated using other methodologies, such as EpCAM-based antibody isolation [12,13], would show similar changes that reflect disease progression and response to chemotherapy.

The morphology of the CTCs may also reflect disease progression and response to chemotherapy. CTC clusters or circulating tumor microemboli, groups of two or more CTCs that are present together in blood, have been shown to increase distal metastases [26]. In this study several CTC clusters were observed in addition to single CTCs, however, because the aim was to rapidly enumerate

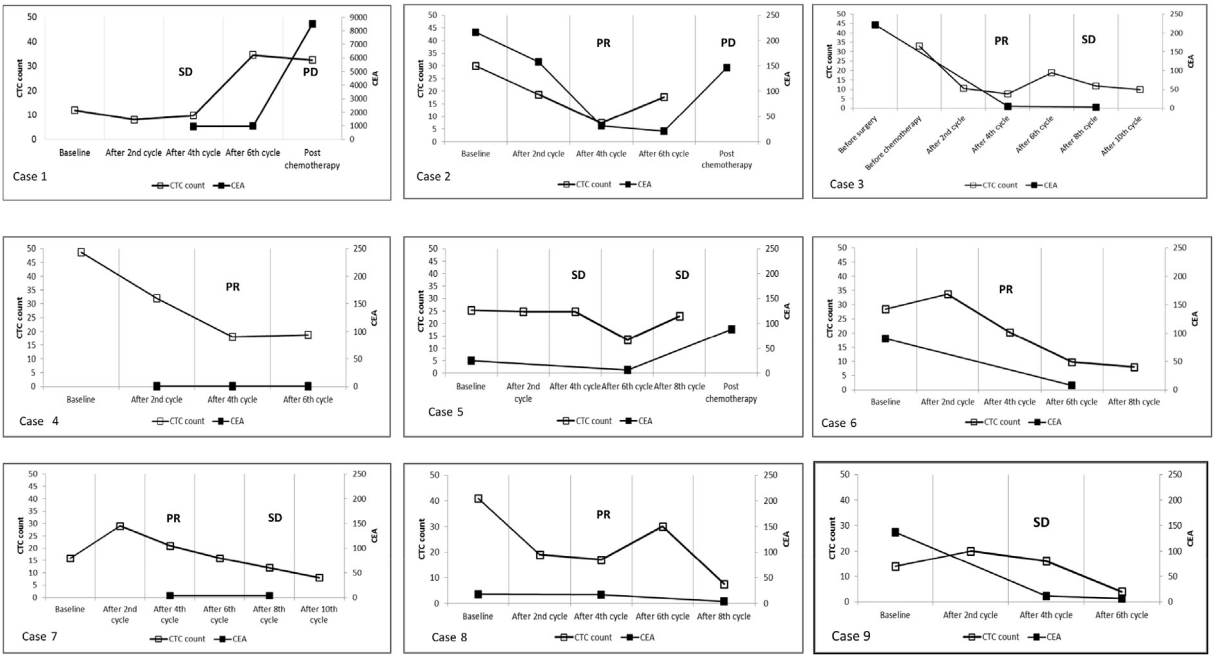

Fig. 1. Longitudinal monitoring of CTC count and CEA levels over the course of chemotherapy. CTC counts (open boxes) are normalized to CTCs per 7.5 ml of blood. CEA levels (closed boxes) are expressed in μg/L. PR = partial response, SD = stable disease, PD = progressive disease.

	PD									SD																			PR															
	Case 1					Case 2				Case 3						Case 5				Case 7						Case 9				Case 4				Case 6					Case 8					
MiRNA	pre	2nd	4th	6th	post	pre	2nd	4th	6th	pre	2nd	4th	6th	8th	10th	pre	2nd	4th	6th	8th	pre	2nd	4th	6th	8th	10th	pre	2nd	4th	6th	pre	2nd	4th	6th	pre	2nd	4th	6th	8th	pre	2nd	4th	6th	8th
miR-15b								3.1						13.0			3.1															3.7	11.6									3.6			
miR-16										4.5				11.1															2.5		6.2										12.0	14.3		
miR-19a					2.8				26.6					4.9																	12.9	4.8													
miR-21	3.0					4.7			40.6					11.0																		8.1			2.4					5.8				
miR-25				2.4					56.6		6.2			4.3		5.9	2.4			4.1			4.1						11.5		56.1	7.0				21.9	3.7			3.0	5.7	3.6	10.8	
miR-30a										5.8																									2.3							5.3		
miR-30d						2.7			2.8	3.2						3.4													19.0		14.5	14.7										14.3	4.5		
miR-34																																			5.2						2.9			
miR-93				9.9																																								
miR-100																																												
miR-106a																																												
miR-125b			8.6																								3.0																	
miR-126										6.0				4.4																	11.4	2.4							2.8			4.6		
miR-185				4.6						3.3	4.0			4.8		5.3							2.5						6.6		5.9	4.8	3.3								3.4	16.5		
miR-192										5.4																																		
miR-196b			6.5												2.7																													
miR-200c			9.5																																4.0									
miR-202				7.7																																								
miR-215			8.4							4.3																	4.6															6.3		
miR-221	2.4		3.2	6.2		3.0			6.5							10.4			3.8				9.8						8.1		8.9	8.2			3.2	6.0	3.4			2.6		2.7	17.6	
miR-222														3.4									14.1								3.4	11.8	2.7										
miR-324-5p	3.8			49.0					3.8	4.2	15.3				2.9																2.3	4.7												
miR-744																																							2.8					
miR-601																											6.4																	

Fig. 2. Heatmap of CTC-associated miRNA expression in metastatic colorectal cancer patients over the course of oxaliplatin-based chemotherapy. Empty squares indicate no expression of the miRNA. Shaded squares indicate expression of the miRNA in the patient at the particular time-point. Darker shade indicates higher miRNA expression. PR = partial response, SD = stable disease, PD = progressive disease. The time-points are indicated as pre = baseline, before chemotherapy, 2nd = after 2nd cycle of chemotherapy, 4th = after 4th cycle of chemotherapy, 6th = after 6th cycle of chemotherapy, 8th = after 8th cycle of chemotherapy, 10th = after 10th cycle of chemotherapy, and post = post chemotherapy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the CTCs to ensure viable cells for RNA extraction, the morphology of the CTCs were not analyzed in detail. It would be of significance to determine whether the presence of CTC clusters versus single CTCs may influence disease progression, response to chemotherapy, or miRNA expression. Vimentin-positive CTCs detected by a size-based platform have been shown to predict worse prognosis in advanced colorectal cancer patients during chemotherapy [27]. It would also be of interest to examine whether vimentin-positive CTCs behave differently from vimentin-negative CTCs.

In this study, CTC-associated miRNA expression did not appear to correlate with CTC count and exhibited inter-individual heterogeneity. We observed very high levels of miRNA expression by CTCs at certain time-points and no miRNA expression at other time-points (Fig. 2). This observation suggests that the expression of miRNAs by CTCs may be a transient phenomenon, possibly due to rapid synthesis and/or degradation of miRNA. Although the observed transient expression of CTC-associated miRNAs may make them unsuitable biomarkers for monitoring disease progression and/or response to therapy in the clinical setting, we have identified certain miRNAs that are highly expressed by CTCs at certain time-points (Fig. 2). This information may be useful to shed light on the function of these miRNAs in the biology of cancer and metastasis.

miR-25 showed the highest expression in cases 2, 6 and 8 at time-points when the CTC counts increased (Fig. 2). miR-25 belongs to the miR-92a family of highly conserved miRNAs with three paralog clusters, miR-17-92, miR-106a-363, and miR-106b-25, known for their roles in tumorigenesis and metastasis [28]. Increased miR-25 expression has been found in other studies to be increased in colorectal cancer and associated with poor prognosis [29]. miR-21 was also highly elevated in case 2 when CTC counts increased before clinical confirmation of progressive disease (Figs. 1

and 2). miR-21 is a well-known oncomiRNA and has been reviewed to be a promising biomarker in the diagnosis and prognosis of colorectal cancer [30,38]. miR-19a also showed very high expression in case 2 (after chemotherapy) and case 4 (before chemotherapy) (Figs. 1 and 2). miR-19a belongs to the miR-17-92 cluster of the miR-92a family [28] and has been shown to be associated with colorectal cancer proliferation [31] and metastasis [32]. miR-324–5p was observed to be highly expressed in case 1 when CTC count increased (Figs. 1 and 2). miR-324 has been less well studied in cancer, with one report that miR-324–5p reduced growth and invasion of colorectal cancer [33]. miR-34 [43], miR-93 [44] and miR-100 [47] have been reported to be downregulated in metastatic colorectal cancer; these miRNAs were not highly expressed in the CTCs of most patients (Fig. 2). Downregulation of miR-126 has been associated with poor prognosis [53]. In our study miR-126 was expressed in CTCs from patients with partial response or stable disease but not from patients with progressive disease (Fig. 2). Functional studies need to be performed in order to understand the molecular mechanisms and dynamism of expression of these miRNAs in chemotherapy response/disease progression in metastatic colorectal cancer.

There have been only a few studies on miRNAs expressed by CTCs [13,19]. Sieuwerts and colleagues identified 28 miRNAs to be measured with more than 10-fold higher expression in CTCs from metastatic breast cancer patients relative to leukocytes [13]. Some of these miRNAs included miR-100, miR-125b, miR-200c, and miR-34a [13]. In this study, these same 4 miRNAs did not appear to be very highly expressed at most time-points in the CTCs from the metastatic colorectal cancer patients (Fig. 2), suggesting that the miRNAs expressed by breast and colorectal cancer CTCs may be different. Markou and colleagues found miR-21, miR-146a, miR-200c and miR-210 to be expressed in CTCs from breast cancer patients, however none of the miRNAs correlated with overall survival [19]. Taken together, these and our present study suggest that cancer-related miRNAs are expressed in CTCs and may differ for different cancer types.

An advantage of our study is that we have measured CTC changes over multiple time-points over the course of chemotherapy, thus allowing better resolution of dynamism exhibited in CTC count over the treatment duration. To our knowledge, this is the first study demonstrating longitudinal monitoring of CTC-associated miRNA expression in colorectal cancer. A limitation of this study is that due to limited patient CTC sample, we were unable to perform technical replication and validation of miRNA analyses. Another limitation is that this is a small study, with only 9 patients; therefore our results need to be replicated in larger studies. Although we have studied only 9 patients, the quality of data from each patient obtained by longitudinal monitoring at multiple time-points helps to derive better resolution of the dynamism of CTC and CTC-related molecular changes involved, which may have implications for diagnosis and treatment [15].

Conclusion

In this pilot study, we have studied the changes in CTC counts and CTC-associated miRNAs over the course of chemotherapy in 9 metastatic colorectal cancer patients. Our results suggest that longitudinal monitoring of the dynamic CTC changes over the course of the disease and treatment in metastatic colorectal cancer may be useful in predicting patient response and disease progression. These results need to be validated in larger studies.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Author contributions

KT, SML, ESCK, WPY, and ASLP conceptualized and designed the study. MVMB, WKC, TS, WPY, ASLP recruited the patients for the study. KT, PVC, JT, ZZK performed the CTC and miRNA experiments and data analyses. KT, MVMB, WPY and ASLP obtained clinical-pathological variables for correlation. KT and ASLP prepared the manuscript. SML, ESCK, WKC, and WPY reviewed the manuscript. All authors approved of the final version to be submitted.

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References

[1] R. Siegel, C. Desantis, A. Jemal, Colorectal cancer statistics, 2014, CA A Cancer J. Clin. 64 (2014) 104–117, https://doi.org/10.3322/caac.21220.
[2] H.J. Schmoll, E. Van Cutsem, A. Stein, V. Valentini, B. Glimelius, K. Haustermans, B. Nordlinger, C.J. van de Velde, J. Balmana, J. Regula, I.D. Nagtegaal, R.G. Beets-Tan, D. Arnold, et al., ESMO Consensus Guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making, Ann. Oncol. 23 (2012) 2479–2516, https://doi.org/10.1093/annonc/mds236.
[3] J. Cassidy, J. Tabernero, C. Twelves, R. Brunet, C. Butts, T. Conroy, F. Debraud, A. Figer, J. Grossmann, N. Sawada, P. Schöffski, A. Sobrero, E. Van Cutsem, et al., XELOX (capecitabine plus oxaliplatin): active first-line therapy for patients with metastatic colorectal cancer, J. Clin. Oncol. 22 (2004) 2084–2091, https://doi.org/10.1200/JCO.2004.11.069.
[4] Y.H. Li, H.Y. Luo, F.H. Wang, Z.Q. Wang, M.Z. Qiu, Y.X. Shi, X.J. Xiang, X.Q. Chen, Y.J. He, R.H. Xu, Phase II study of capecitabine plus oxaliplatin (XELOX) as first-line treatment and followed by maintenance of capecitabine in patients with metastatic colorectal cancer, J. Canc. Res. Clin. Oncol. 136 (2010) 503–510, https://doi.org/10.1007/s00432-009-0682-5.
[5] R.M. Goldberg, D.J. Sargent, R.F. Morton, C.S. Fuchs, R.K. Ramanathan, S.K. Williamson, B.P. Findlay, H.C. Pitot, S.R. Alberts, A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer, J. Clin. Oncol. 22 (2004) 23–30, https://doi.org/10.1200/JCO.2004.09.046.
[6] S. Shiovitz, W.M. Grady, Molecular markers predictive of chemotherapy response in colorectal cancer, Curr. Gastroenterol. Rep. 17 (2015) 431, https://doi.org/10.1007/s11894-015-0431-7.
[7] A.R. Sepulveda, S.R. Hamilton, C.J. Allegra, W. Grody, A.M. Cushman-Vokoun, W.K. Funkhouser, S.E. Kopetz, C. Lieu, N.M. Lindor, B.D. Minsky, F.A. Monzon, D.J. Sargent, V.M. Singh, J. Willis, J. Clark, C. Colasacco, R.B. Rumble, R. Temple-Smolkin, C.B. Ventura, J.A. Nowak, Molecular biomarkers for the evaluation of colorectal cancer: guideline from the american society for clinical pathology, college of american pathologists, association for molecular pathology, and the american society of clinical oncology, J. Clin. Oncol. 35 (2017) 1453–1486, https://doi.org/10.1200/JCO.2016.71.9807.
[8] E. Van Cutsem, C.H. Köhne, E. Hitre, J. Zaluski, C.R. Chang Chien, A. Makhson, G. D'Haens, T. Pintér, R. Lim, G. Bodoky, J.K. Roh, G. Folprecht, P. Ruff, C. Stroh, S. Tejpar, M. Schlichting, J. Nippgen, P. Rougier, Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer, N. Engl. J. Med. 360 (2009) 1408–1417, https://doi.org/10.1056/NEJMoa0805019.
[9] H. Hurwitz, L. Fehrenbacher, W. Novotny, T. Cartwright, J. Hainsworth, W. Heim, J. Berlin, A. Baron, S. Griffing, E. Holmgren, N. Ferrara, G. Fyfe, B. Rogers, R. Ross, F. Kabbinavar, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer, N. Engl. J. Med. 350 (2004) 2335–2342, https://doi.org/10.1056/NEJMoa032691.
[10] S. Maheswaran, D.A. Haber, Circulating tumor cells: a window into cancer biology and metastasis, Curr. Opin. Genet. Dev. 20 (2010) 96–99, https://doi.org/10.1016/j.gde.2009.12.002.
[11] V. Plaks, C.D. Koopman, Werb Z. Cancer, Circulating tumor cells, Science 341 (2013) 1186–1188, https://doi.org/10.1126/science.1235226.
[12] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben, G.V. Doyle, W.J. Allard, L.W. Terstappen, D.F. Hayes, Circulating

tumor cells, disease progression, and survival in metastatic breast cancer, N. Engl. J. Med. 351 (2004) 781–791, https://doi.org/10.1056/NEJMoa040766.
[13] A.M. Sieuwerts, B. Mostert, J. Bolt-de Vries, D. Peeters, F.E. de Jongh, J.M.L. Stouthard, L.Y. Dirix, P.A. van Dam, A. Van Galen, V. de Weerd, J. Kraan, P. van der Spoel, R. Ramírez-Moreno, et al., mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients, Clin. Canc. Res. 17 (2011) 3600–3618, https://doi.org/10.1158/1078-0432.
[14] S. Maheswaran, L.V. Sequist, S. Nagrath, L. Ulkus, B. Brannigan, C.V. Collura, E. Inserra, S. Diederichs, A.J. Iafrate, D.W. Bell, S. Digumarthy, A. Muzikansky, D. Irimia, et al., Detection of mutations in EGFR in circulating lung-cancer cells, N. Engl. J. Med. 359 (2008) 366–377, https://doi.org/10.1056/NEJMoa0800668.
[15] S.M. Leong, K.M. Tan, H.W. Chua, D. Tan, D. Fareda, S. Osmany, M.H. Li, S. Tucker, E.S. Koay, Sampling circulating tumor cells for clinical benefits: how frequent? J. Hematol. Oncol. 8 (2015) 75, https://doi.org/10.1186/s13045-015-0174-9.
[16] L.N. Harris, N. Ismaila, L.M. McShane, F. Andre, D.E. Collyar, A.M. Gonzalez-Angulo, E.H. Hammond, N.M. Kuderer, M.C. Liu, R.G. Mennel, C. Van Poznak, R.C. Bast, D.F. Hayes, American society of clinical oncology. Use of biomarkers to guide decisions on adjuvant systemic therapy for women with early-stage invasive breast cancer: american society of clinical oncology clinical practice guideline, J. Clin. Oncol. 34 (2016) 1134–1150, https://doi.org/10.1200/JCO.2015.65.2289.
[17] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233, https://doi.org/10.1016/j.cell.2009.01.002.
[18] S.A. Waldman, A. Terzic, Translating microRNA discovery into clinical biomarkers in cancer, J. Am. Med. Assoc. 297 (2007) 1923, https://doi.org/10.1001/jama.297.17.1923.
[19] A. Markou, M. Zavridou, I. Sourvinou, G. Yousef, S. Kounelis, N. Malamos, V. Georgoulias, E. Lianidou, Direct comparison of metastasis-related miRNAs expression levels in circulating tumor cells, corresponding plasma, and primary tumors of breast cancer patients, Clin. Chem. 62 (2016) 1002–1011, https://doi.org/10.1373/clinchem.2015.253716.
[20] S.M. Leong, K.M. Tan, H.W. Chua, M.C. Huang, W.C. Cheong, M.H. Li, S. Tucker, E.S. Koay, Paper-based microRNA expression profiling from plasma and circulating tumor cells, Clin. Chem. 63 (2017) 731–741, https://doi.org/10.1373/clinchem.2016.264432.
[21] B. Kosmo, K.W. Lee, MSb Masroni, S. Leong, W.E. Ong, H.L. Wong, Y.Y. Chang, E.S. Koay, S.M. Leong, K. Tan, MicroRNA profiling of oxaliplatin-resistant HCT116 colorectal cancer cells, Oncomedicine 2 (2017) 111–120, https://doi.org/10.7150/oncm.20045.
[22] G. Wan, Q.E. Lim, H.P. Too, High-performance quantification of mature microRNAs by real-time RT-PCR using deoxyuridine-incorporated oligonucleotides and hemi-nested primers, RNA 16 (2010) 1436–1445, https://doi.org/10.1261/rna.2001610.
[23] E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, et al., New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1), Eur. J. Canc. 45 (2009) 228–247, https://doi.org/10.1016/j.ejca.2008.10.026.
[24] L.S. Lim, M. Hu, M.C. Huang, W.C. Cheong, A.T.L. Gan, X.L. Looi, S.M. Leong, E.S. Koay, M.H. Li, Microsieve lab-chip device for rapid enumeration and fluorescence in situ hybridization of circulating tumor cells, Lab a Chip 12 (2012) 4388–4396, https://doi.org/10.1039/c2lc20750h.
[25] M. Yu, A. Bardia, B.S. Wittner, S.L. Stott, M.E. Smas, D.T. Ting, S.J. Isakoff, J.C. Ciciliano, M.N. Wells, A.M. Shah, K.F. Concannon, M.C. Donaldson, L.V. Sequist, et al., Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition, Science 339 (2013) 580–584, https://doi.org/10.1126/science.1228522.
[26] Y. Hong, F. Fang, Q. Zhang, Circulating tumor cell clusters: what we know and what we expect (Review), Int. J. Oncol. 49 (2016) 2206–2216, https://doi.org/10.3892/ijo.2016.3747.
[27] D. Zhang, L. Zhao, P. Zhou, H. Ma, F. Huang, M. Jin, X. Dai, X. Zheng, S. Huang, T. Zhang, Circulating tumor microemboli (CTM) and vimentin+ circulating tumor cells (CTCs) detected by a size-based platform predict worse prognosis in advanced colorectal cancer patients during chemotherapy, Canc. Cell Int. 17 (2017) 6, https://doi.org/10.1186/s12935-016-0373-7.
[28] M. Li, X. Guan, Y. Sun, J. Mi, X. Shu, F. Liu, C. Li, miR-92a family and their target genes in tumorigenesis and metastasis, Exp. Cell Res. 323 (2014) 1–6, https://doi.org/10.1016/j.yexcr.2013.12.025.
[29] X. Li, C. Yang, X. Wang, J. Zhang, R. Zhang, R. Liu, The expression of miR-25 is increased in colorectal cancer and is associated with patient prognosis, Med. Oncol. 31 (2014) 781, https://doi.org/10.1007/s12032-013 0781–0787.
[30] Q. Peng, X. Zhang, M. Min, L. Zou, P. Shen, Y. Zhu, The clinical role of microRNA-21 as a promising biomarker in the diagnosis and prognosis of colorectal cancer: a systematic review and meta-analysis, Oncotarget 8 (2017) 44893–44909, https://doi.org/10.18632/oncotarget.16488.
[31] Y. Liu, R. Liu, F. Yang, R. Cheng, X. Chen, S. Cui, Y. Gu, W. Sun, C. You, Z. Liu, F. Sun, Y. Wang, Z. Fu, C. Ye, C. Zhang, J. Li, X. Chen, miR-19a promotes colorectal cancer proliferation and migration by targeting TIA1, Mol. Canc. 16 (2017) 53, https://doi.org/10.1186/s12943-017-0625-8.
[32] L. Huang, X. Wang, C. Wen, X. Yang, M. Song, J. Chen, C. Wang, B. Zhang, L. Wang, A. Iwamoto, J. Wang, H. Liu, Hsa-miR-19a is associated with lymph metastasis and mediates the TNF-α induced epithelial-to-mesenchymal transition in colorectal cancer, Sci. Rep. 5 (2015) 13350, https://doi.org/10.1038/srep13350.
[33] W.T. Kuo, S.Y. Yu, S.C. Li, H.C. Lam, H.T. Chang, W.S. Chen, C.Y. Yeh, S.F. Hung, T.C. Liu, T. Wu, C.C. Yu, K.W. Tsai, MicroRNA-324 in human cancer: miR-324-5p and miR-324-3p have distinct biological functions in human cancer, Anticancer Res. 36 (2016) 5189–5196, https://doi.org/10.21873/anticanres.11089.
[34] J. Li, Y. Chen, X. Guo, L. Zhou, Z. Jia, Y. Tang, L. Lin, W. Liu, C. Ren, Inhibition of miR-15b decreases cell migration and metastasis in colorectal cancer, Tumour Biol. 37 (2016) 8765–8773, https://doi.org/10.1007/s13277-015-4396-9.
[35] M.I. Aslam, J. Venkatesh, J.S. Jameson, K. West, J.H. Pringle, B. Singh, Identification of high-risk Dukes B colorectal cancer by microRNA expression profiling: a preliminary study, Colorectal Dis. 17 (2015) 578–588, https://doi.org/10.1111/codi.12886.
[36] M.A. Diamantopoulos, C.K. Kontos, D. Kerimis, I.N. Papadopoulos, A. Scorilas, Upregulated miR-16 expression is an independent indicator of relapse and poor overall survival of colorectal adenocarcinoma patients, Clin. Chem. Lab. Med. 55 (2017) 737–747, https://doi.org/10.1515/cclm-2016-0756.
[37] Q. Chen, H.W. Xia, X.J. Ge, Y.C. Zhang, Q.L. Tang, F. Bi, Serum miR-19a predicts resistance to FOLFOX chemotherapy in advanced colorectal cancer cases, Asian Pac. J. Cancer Prev. APJCP 14 (2013) 7421–7426, https://doi.org/10.7314/APJCP.2013.14.12.7421.
[38] X. Xia, B. Yang, X. Zhai, X. Liu, K. Shen, Z. Wu, J. Cai, Prognostic role of microRNA-21 in colorectal cancer: a meta-analysis, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0080426 e80426.
[39] M. Liu, F. Huang, D. Zhang, J. Ju, X.B. Wu, Y. Wang, Y. Wang, Y. Wu, M. Nie, Z. Li, C. Ma, X. Chen, J.Y. Zhou, R. Tan, B.L. Yang, K. Zen, C.Y. Zhang, Y.G. Chen, Q. Zhao, Heterochromatin protein HP1γ promotes colorectal cancer progression and is regulated by miR-30a, Canc. Res. 75 (2015) 4593–4604, https://doi.org/10.1158/0008-5472.CAN-14-3735.
[40] L. Liu, T. Meng, Q.S. Wang, H.Z. Jin, Z.Q. Sun, B. Jin, F. Fang, H.J. Wang, Association of Beclin-1 and microRNA-30a expression with the severity and treatment response of colorectal cancer, Genet. Mol. Res. 15 (2016), https://doi.org/10.4238/gmr.15027704.
[41] A. Nijhuis, H. Thompson, J. Adam, A. Parker, L. Gammon, A. Lewis, J.G. Bundy, T. Soga, A. Jalaly, D. Propper, R. Jeffery, N. Suraweera, S. McDonald, M.A. Thaha, R. Feakins, R. Lowe, C.L. Bishop, A. Silver, Remodelling of microRNAs in colorectal cancer by hypoxia alters metabolism profiles and 5-fluorouracil resistance, Hum. Mol. Genet. 26 (2017) 1552–1564, https://doi.org/10.1093/hmg/ddx059.
[42] T. Vu, P.K. Datta, Regulation of EMT in colorectal cancer: a culprit in vetastasis, Cancers 9 (2017), https://doi.org/10.3390/cancers9120171. E171.
[43] S. Roy, E. Levi, A.P. Majumdar, F.H. Sarkar, Expression of miR-34 is lost in colon cancer which can be re-expressed by a novel agent CDF, J. Hematol. Oncol. 5 (2012) 58, https://doi.org/10.1186/1756-8722-5-58.
[44] J. Zhou, Y. Zhou, B. Yin, W. Hao, L. Zhao, W. Ju, C. Bai, 5-Fluorouracil and oxaliplatin modify the expression profiles of microRNAs in human colon cancer cells in vitro, Oncol. Rep. 23 (2010) 121–128, https://doi.org/10.3892/or_00000613.
[45] Z.G. Xiao, Z.S. Deng, Y.D. Zhang, Y. Zhang, Z.C. Huang, Clinical significance of microRNA-93 downregulation in human colon cancer, Eur. J. Gastroenterol. Hepatol. 25 (2013) 296–301, https://doi.org/10.1097/MEG.0b013e32835c0.
[46] S. Wang, J. Xiang, Z. Li, S. Lu, J. Hu, X. Gao, L. Yu, L. Wang, J. Wang, Y. Wu, Z. Chen, H. Zhu, A plasma microRNA panel for early detection of colorectal cancer, Int. J. Canc. 136 (2015) 152–161, https://doi.org/10.1002/ijc.28136.
[47] P. Chen, Q. Xi, Q. Wang, P. Wei, Downregulation of microRNA-100 correlates with tumor progression and poor prognosis in colorectal cancer, Med. Oncol. 31 (2014) 235, https://doi.org/10.1007/s12032-014-0235-x.
[48] B. Feng, T.T. Dong, L.L. Wang, H.M. Zhou, H.C. Zhao, F. Dong, M.H. Zheng, Colorectal cancer migration and invasion initiated by microRNA-106a, PLoS One 7 (2012), e43452, https://doi.org/10.1371/journal.pone.0043452.
[49] Y. Koga, N. Yamazaki, Y. Yamamoto, S. Yamamoto, N. Saito, Y. Kakugawa, Y. Otake, M. Matsumoto, Y. Matsumura, Fecal miR-106a is a useful marker for colorectal cancer patients with false-negative results in immunochemical fecal occult blood test, Cancer Epidemiol. Biomark. Prev. 22 (2013) 1844–1852, https://doi.org/10.1158/1055-9965.
[50] N. Nishida, T. Yokobori, K. Mimori, T. Sudo, F. Tanaka, K. Shibata, H. Ishii, Y. Doki, H. Kuwano, M. Mori, MicroRNA miR-125b is a prognostic marker in human colorectal cancer, Int. J. Oncol. 38 (2011) 1437–1443, https://doi.org/10.3892/ijo.2011.969.
[51] X. Yu, W. Shi, Y. Zhang, X. Wang, S. Sun, Z. Song, M. Liu, Q. Zeng, S. Cui, X. Qu, CXCL12/CXCR4 axis induced miR-125b promotes invasion and confers 5-fluorouracil resistance through enhancing autophagy in colorectal cancer, Sci. Rep. 7 (2017) 42226, https://doi.org/10.1038/srep42226.
[52] X. Zhang, X. Ma, H. An, C. Xu, W. Cao, W. Yuan, J. Ma, Upregulation of microRNA-125b by G-CSF promotes metastasis in colorectal cancer, Oncotarget 8 (2017) 50642–50654, https://doi.org/10.18632/oncotarget.16892.
[53] W. Huang, J. Lin, H. Zhang, miR-126: a novel regulator in colon cancer, Biomed Rep 4 (2016) 131–134, https://doi.org/10.3892/br.2015.549.
[54] P. Akçakaya, S. Ekelund, I. Kolosenko, S. Caramuta, D.M. Ozata, H. Xie, U. Lindforss, H. Olivecrona, W.O. Lui, miR-185 and miR-133b deregulation is associated with overall survival and metastasis in colorectal cancer, Int. J. Oncol. 39 (2011) 311–318, https://doi.org/10.3892/ijo.2011.1043.
[55] M. Liu, N. Lang, X. Chen, Q. Tang, S. Liu, J. Huang, Y. Zheng, F. Bi, miR-185 targets RhoA and Cdc42 expression and inhibits the proliferation potential of

human colorectal cells, Canc. Lett. 301 (2011) 151–160, https://doi.org/10.1016/j.canlet.2010.11.009.
[56] L. Geng, A. Chaudhuri, G. Talmon, J.L. Wisecarver, C. Are, M. Brattain, J. Wang, MicroRNA-192 suppresses liver metastasis of colon cancer, Oncogene 33 (2014) 5332–5340, https://doi.org/10.1038/onc.2013.478.
[57] J. Yang, D. Ma, A. Fesler, H. Zhai, A. Leamniramit, W. Li, S. Wu, J. Ju, Expression analysis of microRNA as prognostic biomarkers in colorectal cancer, Oncotarget 8 (2016) 52403–52412, https://doi.org/10.18632/oncotarget.14175.
[58] J. Ge, Z. Chen, R. Li, T. Lu, G. Xiao, Upregulation of microRNA-196a and microRNA-196b cooperatively correlate with aggressive progression and unfavorable prognosis in patients with colorectal cancer, Canc. Cell Int. 14 (2014) 128, https://doi.org/10.1186/s12935-014-0128-2.
[59] D. Ren, B. Lin, X. Zhang, Y. Peng, Z. Ye, Y. Ma, Y. Liang, L. Cao, X. Li, R. Li, L. Sun, Q. Liu, J. Wu, K. Zhou, J. Zeng, Maintenance of cancer stemness by miR-196b-5p contributes to chemoresistance of colorectal cancer cells via activating STAT3 signaling pathway, Oncotarget 8 (2017) 49807–49823, https://doi.org/10.18632/oncotarget.17971.
[60] W. Li, J. Chang, D. Tong, J. Peng, D. Huang, W. Guo, W. Zhang, J. Li, Differential microRNA expression profiling in primary tumors and matched liver metastasis of patients with colorectal cancer, Oncotarget 8 (2017) 35783–35791, https://doi.org/10.18632/oncotarget.16206.
[61] J. Chen, W. Wang, Y. Zhang, T. Hu, Y. Chen, The roles of miR-200c in colon cancer and associated molecular mechanisms, Tumour Biol. 35 (2014) 6475–6483, https://doi.org/10.1007/s13277-014-1860-x.
[62] Y. Toiyama, K. Hur, K. Tanaka, Y. Inoue, M. Kusunoki, C.R. Boland, A. Goel, Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer, Ann. Surg. 259 (2014) 735–743, https://doi.org/10.1097/SLA.0b013e3182a6909d.
[63] Y. Zhu, Q. Peng, Y. Lin, L. Zou, P. Shen, F. Chen, M. Min, L. Shen, J. Chen, B. Shen, Identification of biomarker microRNAs for predicting the response of colorectal cancer to neoadjuvant chemoradiotherapy based on microRNA regulatory network, Oncotarget 8 (2017) 2233–2248, https://doi.org/10.18632/oncotarget.13659.
[64] M. Karaayvaz, T. Pal, B. Song, C. Zhang, P. Georgakopoulos, S. Mehmood, S. Burke, K. Shroyer, J. Ju, Prognostic significance of miR-215 in colon cancer, Clin. Colorectal Canc. 10 (2011) 340–347.
[65] J. Qin, M. Luo, MicroRNA-221 promotes colorectal cancer cell invasion and metastasis by targeting RECK, FEBS Lett. 588 (2014) 99–104, https://doi.org/10.1016/j.febslet.2013.11.014.
[66] M. Sarlinova, M. Halasa, D. Mistuna, L. Musak, R. Iliev, O. Slaby, J. Mazuchova, V. Valentova, L. Plank, E. Halasova, miR-21, miR-221 and miR-150 are deregulated in peripheral blood of patients with colorectal cancer, Anticancer Res. 36 (2016) 5449–5454, https://doi.org/10.21873/anticanres.11124.
[67] T.O. Yau, C.W. Wu, Y. Dong, C.M. Tang, S.S. Ng, F.K. Chan, J.J. Sung, J. Yu, microRNA-221 and microRNA-18a identification in stool as potential biomarkers for the non-invasive diagnosis of colorectal carcinoma, Br. J. Canc. 111 (2014) 1765–1771, https://doi.org/10.1038/bjc.2014.484.
[68] Q. Wang, Z. Huang, S. Ni, X. Xiao, Q. Xu, L. Wang, D. Huang, C. Tan, W. Sheng, X. Du, Plasma miR-601 and miR-760 are novel biomarkers for the early detection of colorectal cancer, PLoS One 7 (2012), e44398, https://doi.org/10.1371/journal.pone.0044398.