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MicroRNA as Biomarkers in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the worlds leading causes of cancer-related deaths in both men and women. Nearly 1.4 million new cases were reported in 2012 and over half of the cases ended in death. High mortality rates are largely due to late diagnosis, at a stage when treatment efficacy is limited. Current methods for CRC detection and evaluation of prognosis and treatment response are compromised by limited sensitivity and specificity. MicroRNA (miRNA) are small RNA molecules that regulate gene expression by targeting messenger RNA (mRNA) transcripts. Aberrantly expressed miRNAs have been implicated as important players in CRC genesis, however, the functional role for many deregulated miRNAs in CRC are unknown. MiRNA is found in tissue and body fluids and have been proposed as potential non-invasive biomarkers in CRC.

Total small RNA were isolated from serum of rectal cancer patients and prepared for high throughput sequencing. Patient data were collected from medical records to analyze the correlation between miRNA expression levels and clinical and tumor characteristics in order to identify candidate miRNAs. The effects of deregulated miRNAs in CRC tissue on cell growth were tested by transfection of miRNA mimics and inhibitors in the SW480 CRC cell line and measured by cell viability.

Sequencing of isolated RNA identified 498 mature miRNAs and 3758 isomiRs. Several miRNAs and corresponding isomiRs (sequence variants) were significantly associated with metastasis at diagnosis, CEA levels and/or overall survival (miR-320a-e, miR-10a-5p, miR-1246, let-7b-5p, miR-200c-3p and miR-29a-3p). Serum levels of miRNAs miR-10b-5p, miR-215-5p and miR-150-5p, and isomiRs of miR-125b-5p and miR-30a-5p were significantly changed in CRC patients depending on whether patients had received preoperative treatment or not before serum was collected. Levels of two miRNAs, miR-451a and miR-877-5p, correlated with hemoglobin levels.

In the functional assay, several miRNA inhibitors had a significant growth inhibitory effect on CRC cells (e.g. miR-183, miR-182, miR-96, miR-31-5p and miR-18a-3p). It seemed that some miRNA mimics, at least in some part, caused a decrease in CRC cell growth, but were not significant in our experiment.

In conclusion, the present study showed that several aberrantly expressed miRNAs in CRC tissue play important roles in growth of CRC cells, and that several miRNAs, including miR-320a-e, miR-10a-5p, miR-1246 and let-7b-5p, and miR-10b-5p, miR-150-5p, miR-215-5p and miR-125b-5p are potential prognostic and predictive biomarkers in CRC. Our findings also suggest that isomiRs may provide an additional layer to CRC biomarkers beyond mature miRNAs. Validation in large-scale studies and assessment of specificity and sensitivity is needed to determine the clinical application of these biomarkers.

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Abbreviations

CRC	Colorectal cancer
CT scan	Computed tomography scan
MRI	Magnetic resonance imaging
CEA	Carcinoembryonic antigen
FOBT	Fecal occult blood test
MSI	Microsatellite instability
MSS	Microsatellite stability
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
miRNA	microRNA
mRNA	Messenger RNA
miRISC	microRNA-induced silencing complex
AGO2	Argonaute 2 (protein)
UTR	Untranslated region
μL	Micro litre
PCR	Polymerase chain reaction
rpm	Reads per million
cpm	Counts per million
MDS	Multidimensional scaling
NTA	Non-templated nucleotide addition
FC	Fold change
hsa-miR	Human microRNA

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1. Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is the third most common cause of cancer-related deaths worldwide, in both men and women (1). Of the nearly 1.4 million new cases reported in 2012, over half of the cases ended in death. Incidence of CRC is higher in developed regions compared to less developed regions (2), which may reflect an increased exposure to risk factors of CRC, such as smoking, an unhealthy diet and other lifestyle factors (3,4). The opposite is observed for mortality rates and is largely caused by late diagnosis due to only modest or lack of symptoms at an early stage, and thus many patients present with advanced disease and metastases at diagnosis (5). However, more focus on preventive measures such as screening and surveillance, as well as improvements in disease management, has resulted in reduced incidence and mortality in some parts of the world (6).

Colorectal cancer is referred to as cancers that arise in the colon (large intestine) or rectum. Rectal cancer is most often defined as cancers originating within 15 cm form the anal verge (7,8). Development of CRC is a multistep process that involves genetic and epigenetic alterations that are required for cancer initiation and progression. The earliest genetic trigger event is inactivation of the APC (adenomatous polyposis coli) pathway. Mutations in tumor suppressor genes (APC, TP53), oncogenes (KRAS, BRAF, Bcl2, PI3K) and other genes, such as DNA mismatch repair (MMR) genes, accompany the stepwise transition from single crypt lesions to benign adenomatous polyps and finally development of malignant carcinomas (9), known as the adenoma-carcinoma sequence (10-12). The majority (70%) of CRCs arises sporadically, while the remaining 30% represent patients with a familial or inherited form of the disease (13).

1.1.1 Diagnosis

Primary diagnosis of colorectal cancer is based on clinical findings by endoscopic examination and biopsy. Flexible colonoscopy is the gold standard method for diagnosis, allowing examination of the rectum and entire colon, with possibilities of performing biopsies and polyp removal (polypectomy) in the same procedure. When a positive CRC diagnosis is made, CT (computed tomography) scan of thorax and abdomen is performed to

provide accurate localization and stage determination of the tumor and to assess the tumor extent. Additional MRI of rectum and pelvis is recommended for rectal cancer patients. Measurement of CEA (carcinoembryonic antigen) tumor marker levels is recommended to enable disease monitoring (14).

1.1.2 Classification and disease staging

The TNM staging system is the most widely used and recommended system for CRC staging (15). TNM stage is based on the extent of the disease at diagnosis, which provides an important estimation of prognosis in CRC (16). TNM staging includes clinical findings (cTNM) and radiologic imaging (rTNM) prior to diagnosis, and pathological examination of resected tumor specimens or perioperative findings (pTNM, or ypTNM when staging is made after neoadjuvant treatment) (14,17). The T stage describes the depth of invasion of the primary tumor through the layers of the intestinal wall, N stage describes spread to regional lymph nodes, and the M stage describes the occurrence of distant metastases. TNM stages are classified in stage groups (stage I-IV) where increasing stage corresponds to a more advanced disease, e.g. lymph node metastasis (stage III) and metastasis (stage IV) (18).

Based on microscopic features, CRCs are graded in terms of resemblance to the tissue from which it originated and the proportion of gland formation by the tumor (19). Tumor differentiation grade range from highly differentiated tumors with >95% gland formation, to undifferentiated tumors with less than 5% glandular structures. Histopathological differentiation grade is an important prognostic factor in CRC as low differentiation grade is associated with poorer outcome (20,21).

1.1.3 Treatment

According to an annual report from The Norwegian Cancer Registry, an estimated 70% of CRC patients undergo surgical resection as the primary modality of treatment (22), in which the main goal is to remove the entire tumor for curative intent and long-term survival, and to reduce the risk of local recurrence (23). Appropriate treatment is largely based on pathological assessment at diagnosis, extent of the disease and patient comorbidity to ensure final decisions corresponds to the patients needs. Surgery is often sufficient for management

of early-stage disease (stage I-II), whereas more advanced disease (stage III-IV) typically require supplementary radiotherapy and/or chemotherapy.

Preoperative (neoadjuvant) treatement of rectal cancer is used in an attempt to reduce tumor size to ensure complete surgical removal and prevent local recurrence. For patients with advanced disease, long-term radiotherapy combined with chemotherapy (chemoradiotherapy) and delayed surgery is recommended (14). Short-term preoperative radiotherapy with immediate surgery is an alternative in metastatic and elderly patients (24). Postoperative (adjuvant) chemotherapy is aimed at eliminating potential micro-metastases after surgery, and is recommended for patients with non-complete surgical removal, or patients with infiltrating T4 tumors that did not receive preoperative treatment (14)

1.1.4 Early detection and screening

Mean age at diagnosis for rectal cancer is 63 years in men and 65 years in women, whereas in colon cancer the mean age is 69 and 72 years for men and women, respectively. CRC may cause symptoms like anemia, rectal bleeding, changes in bowel habits, weight loss and general malaise. Symptoms often occur at a late stage when treatment efficacy is limited and about 20-30% of all CRC patients are diagnosed with metastatic (IV) disease (5). The prognosis is significantly better for patients diagnosed at an early stage, as the 5-year survival rate declines from nearly 90% in early-stage (stage I-II) disease to ~12% in late-stage disease (III-IV) (25).

Implementation of mass screening programs has shown to reduce incidence and mortality of CRC (26-29). Screening for CRC reduces the cancer burden by allowing early detection of cancerous lesions and removal of precancerous polyps. Colonoscopy and the fecal occult blood test (FOBT) are the most widely used methods for CRC screening (30). FOBT is used to detect blood in stool from bleeding lesions. FOBT is a simpler and less invasive test than colonoscopy but is compromised by limited sensitivity and specificity (31). Colonoscopy is considered the most specific and sensitive CRC detection method (32). However, adenomas ≤1.0 cm have failed to be detected and a miss rate up to 27% has been reported (33-35). Colonoscopy has several disadvantages in that it is an invasive and time-consuming procedure requiring trained personnel . Complete bowel preparation prior to the test is for many patients worse than the actual procedure (36). Highly accurate and minimally invasive

tests are needed to increase people's willingness to participate in CRC screening programs in order to reduce the overall burden of CRC.

1.1.5 Biomarkers in colorectal cancer

The continuing increase in CRC incidence has initiated an extensive search for cancer-related biomarkers and their potential prognostic and predictive value. Despite the effort, still no single markers or combinations of markers have been discovered that are unique to CRC and provide information on disease outcome and treatment response. TNM stage is currently the most important prognostic factor in CRC, in which increasing TNM stage correlates with adverse prognosis (16,23). However, TNM stage is not optimal to predict disease outcome as patients within the same TNM stage may have different prognosis and response to therapy. CEA in serum is the most widely used biomarker in CRC. Elevated levels at diagnosis are associated with increased tumor stage and poor prognosis (37), but the test is compromised by low sensitivity and specificity and high rates of false positive. Indeed, CEA levels tend to be lower in early stage CRC and high levels are found in other types of cancers, non-malignant conditions, and smokers (38-41), making it insufficient for early detection and screening. Despite its disadvantages, CEA is an inexpensive and non-invasive test that is useful to monitor CRC progression and recurrence (42,43). CA (carcinoma antigen) 19-9 tumor marker is often measured in addition to CEA. Elevated levels are associated with advanced-stage disease and adverse prognosis, but the test is limited by low specificity and sensitivity and its clinical usefulness is not clear (44,45).

KRAS oncogene is an important mediator in the RAS/MAPK pathway downstream of EGFR (epidermal growth factor receptor), which is a major regulator of cell proliferation. Mutated KRAS is a frequent event in CRC leading to constitutive signaling and uncontrolled cell growth. Mutational analysis of KRAS is standard in metastatic CRC (mCRC) patients to predict response to anti-EGFR therapy as mutations in KRAS are related to resistance to anti-EGFR drugs (46). However, not all patients with wild type (wt) KRAS are responsive to anti-EGFR therapy (47). Low response rates and application in only a subset of CRC patients makes the clinical efficacy of KRAS as a predictive biomarker suboptimal. BRAF oncogene is another mediator in the RAS/MAPK pathway frequently mutated in CRC and is associated with lack of response in mCRC patients treated with EGFR inhibitors and poor prognosis (48). However, studies have found varying results and in a recent study by Karapetis et al., no

predictive significance for mutated BRAF in response to anti-EGFR therapy in CRC patients were found (49).

Mutations in other cancer-related genes, such as TP53, PI3K and PTEN, and mechanisms leading to genetic instability, including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP), have been proposed as promising biomarkers in CRC (49-51), but more investigation is needed to establish their potential clinical use. Cleary, there is an urgent need for unique and reliable biomarkers that are able to discriminate CRC patients from healthy individuals, as well as different CRC subgroups from each other, that hold prognostic and predictive value.

1.2 MicroRNA

MicroRNA (miRNA) are short single-stranded RNA molecules of ~21 nucleotides (nt) that regulate gene expression at the post-transcriptional level by targeting of messenger RNA (mRNA). MiRNAs is an abundant class of non-coding RNAs (ncRNAs) that have been evolutionarily conserved between vertebrates, invertebrates and plants (52,53).

1.2.1 Biological effect of microRNA regulation

Lee and colleagues were the first to discover miRNAs in 1993 when they identified small RNA molecules that regulated transition between different larval stages in *Caenorhabditis elegans* (*C.elegans*) (54). The *C.elegans lin-4* and *let-7* genes were shown to not encode proteins, but instead short RNA species with complementarity to multiple sites in the 3'UTR (untranslated region) of certain *C.elegans* mRNAs, which lead to reduced mRNA and protein levels (55,56). Since these initial findings, more than 2500 human miRNAs have been identified (miRBase: Release 21, June 2014) and are predicted to regulate more than half of all protein-coding genes (57). The fact that miRNAs are found in most living organisms and that many mRNAs are conserved miRNA targets, illustrates their widespread importance and potential role in a range of biological processes. Indeed, miRNAs have been linked to metabolic processes (58), regulation of the immune system (59), and various aspects of animal development (60).

Even though a single miRNA can regulate multiple targets at the protein level, studies have shown that the overall effect is relatively low (less than twofold) (61,62), suggesting that

miRNAs are involved in fine-tuning protein output. Protein synthesis is one of the most important determinants of phenotype, and the observation that knockouts of single miRNAs only cause moderate phenotypic consequences for an organism, shows a substantial redundancy and robustness between miRNAs (63). Conversely, global loss of miRNA caused by depletion of key components in the miRNA processing machinery can result in early developmental arrest and death (64-66). It is evident that miRNAs play an important role in biological processes and this is further supported by detection of deregulated miRNA expression in various human diseases (67-69).

1.2.2 MicroRNA biogenesis

Mature miRNAs are generated through sequential steps in the miRNA biogenesis pathway (Fig. 1.1). MiRNA genes typically localize to introns of non-coding or protein-coding genes but might also be located within exons and intergenic regions of non-coding genes (70,71). Certain miRNA genes are found in close proximity to each other and these clustered genes are transcribed as polycistronic units (72). MiRNA genes are transcribed by RNA polymerase II (Pol II) as long primary transcripts (pri-miRNA) with one or more characteristic hairpin structures, a 5'cap and polyadenylated 3'end. In the nucleus, pri-miRNAs are cleaved into ~70nt precursor miRNAs (pre-miRNAs) by Drosha and DGCR8, which are core components of the Microprocessor complex. Pre-miRNAs are then transported to the cytoplasm through Exportin 5 for further processing by Dicer1 in complex with TRBP and PACT. Dicer1-mediated cleavage produces ~21nt miRNA duplexes (miRNA-miRNA*) that are loaded onto Argonaute (AGO2) proteins and unwound to generate two mature miRNAs. AGO2 facilitates incorporation of the guide strand (miRNA) into the miRNA-induced silencing complex (miRISC), whereas the passenger strand (miRNA*) is degraded. The guide strand directs the miRISC to complementary target sites within mRNAs, usually in the 3' untranslated region (UTR), to enable regulation of the targets (Reviewed in (73)). Targeting mechanisms and mRNA regulation will be discussed later. Although there is a strong bias towards the guide strands, increasing evidence suggests that the passenger strand can be selected as a functional strand and have substantial regulatory influence (74,75).

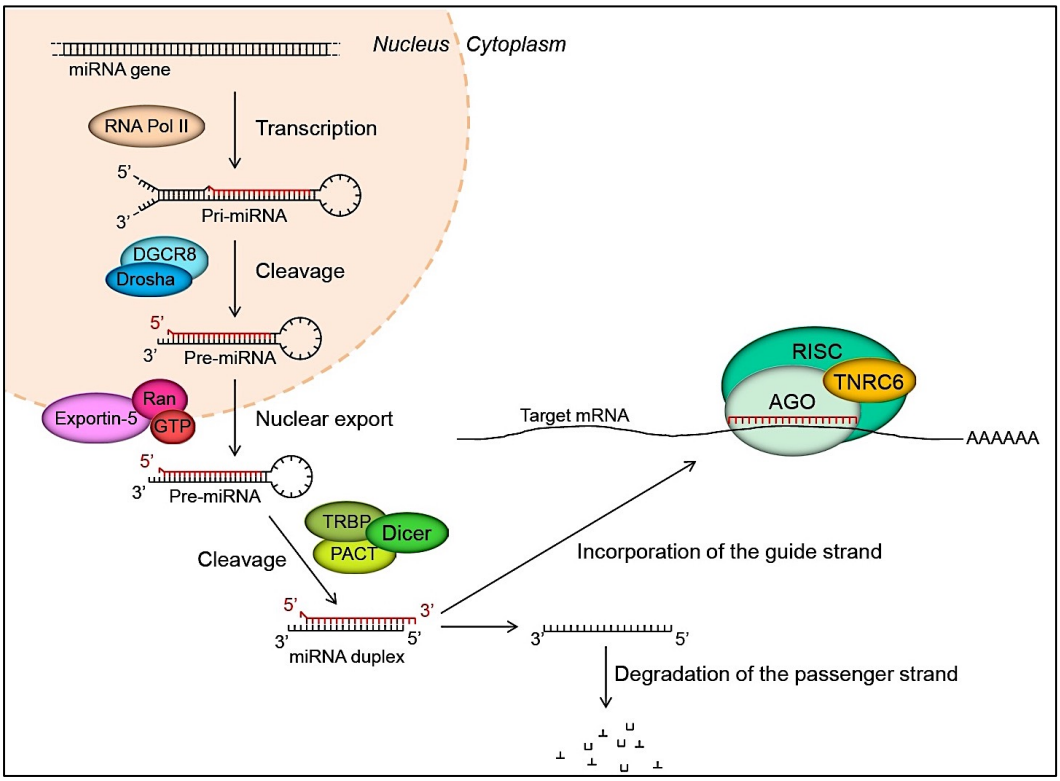


Figure 1.1. The canonical microRNA biogenesis pathway. MicroRNA genes are transcribed by RNA polymerase II to generate primary transcripts (pri-miRNAs). In the initial step, Drosha and DGCR8 of the Microprocessor complex mediate cleavage of the pri-miRNA hairpin structures, thereby generating precursor miRNA (pre-miRNA). Pre-miRNAs are transported to the cytoplasm through Exportin 5 for further cleavage by Dicer1 in complex with TRBP and PACT. Dicer1 mediates cleavage of the dsRNA stem close to the terminal loop of the pre-miRNAs that produces mature ~21nt miRNA duplexs with 2-nt 3'overhangs. The AGO2-bound guide strand is incorporated in the miRISC and directs it to target mRNAs with complementary target sites, whereas the passenger strand is degraded. Figure is based on Winther J et al., Nat Cell Biol, 2009 (76) and modified by Marie Brenner Lundbæk.

In addition to the canonical miRNA biogenesis, alternative pathways for generating mature miRNAs have been described. In the miRtron pathway, Drosha-mediated cleavage of short intronic transcripts is substituted with splicing, but eventually merges with the canonical miRNA biogenesis at the Exportin-5 transport stage (77). Yang and colleagues described another alternative biogenesis pathway, in which generation of mature miRNA is Dicer1-independent. They reported that maturation of miR-451 in humans, mice and zebrafish is independent of Dicer1-mediated cleavage and proposed that the pre-miRNA generated by Drosha-DGCR8 cleavage is too short to act as substrate for Dicer1, and is rather cleaved into mature miRNAs by AGO2 directly (78).

1.2.3 MicroRNA targeting mechanisms and target regulation

Most mammalian miRNAs recognize their targets by pairing of a short sequence (2-7nt) in their 5'region, referred to as the "miRNA seed", to a perfect antisense complementary sequence in the target 3'UTR, known as the "seed match" (79). MiRNA target regulation is associated with four types of seed pairings (Fig. 1.2). These include a 6nt seed match (6mer site) and three canonical target sites containing the seed match flanked by either an adenine (A) across from nt 1 of the miRNA (7mer-A1 site), an additional complementary base match to the miRNA nt 8 (7mer-m8 site), or both (8mer site) (57,80). Atypical sites with complementarity extending beyond the seed have also been identified; sites that is not functional without additional pairing to the miRNA 3'end. Extended complementarity to the 3'end can enhance target recognition (3'supplementary sites) or compensate for single nucleotide bulges or mismatches in the seed (3'complementary sites) (81,82). In cases of (near) perfect complementarity to the entire length of the mRNA, AGO2 can mediate direct cleavage of the target, a mechanism that is more prevalent in plants and only a few examples have been reported in mammals (83,84).

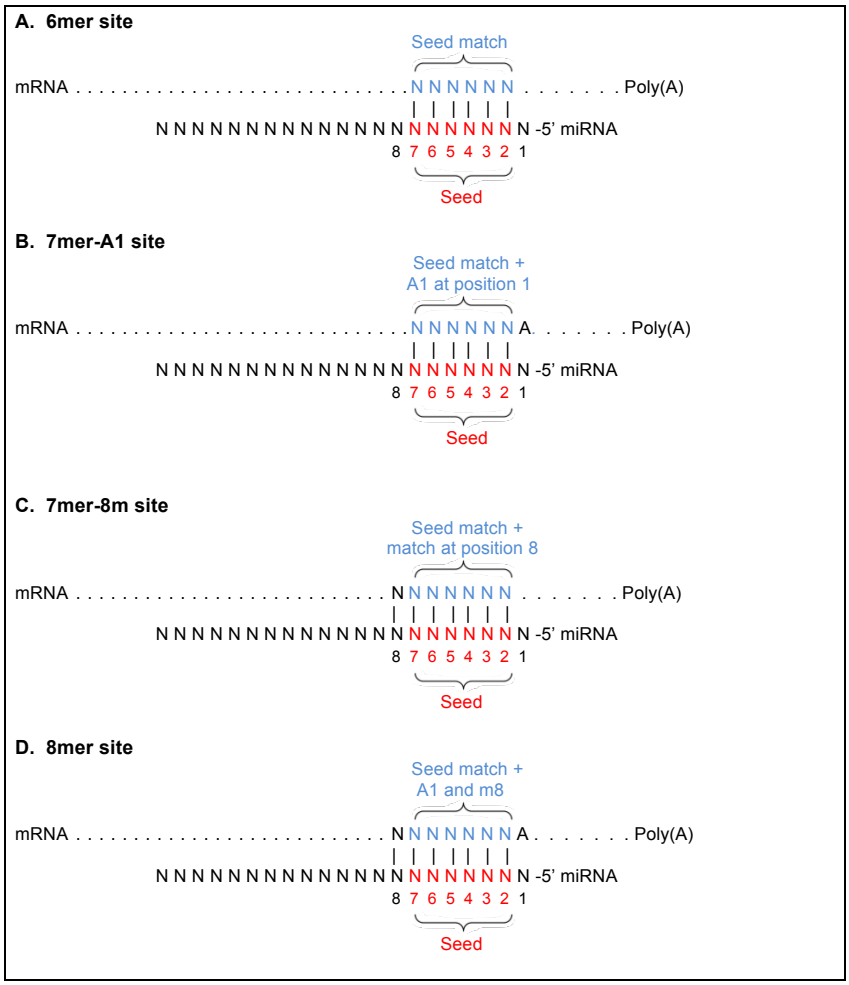


Figure 1.2. MicroRNA target sites. MicroRNAs typically recognize their targets through perfect base-pairing between nt 2-7 of the miRNA ("miRNA seed") and complementary sites in the mRNA 3'UTR ("seed match"). A) 6mer site or "seed match". B-D) Canonical sites contains the seed match and either involve an adenine across from the nucleotide at position 1 of the miRNA (7mer-A1), an additional match to the nucleotide at position 8 of the miRNA (7mer-8m), or both (8mer). Illustration is modified from Bartel DP, Cell, 2009 (81).

MiRNA regulation efficacy is determined by the miRNA-target interactions, such as the location and type of target site, and the number of target sites within an mRNA (85). Most target sites are found in the mRNA 3'UTR, and during the course of evolution it seems that housekeeping genes have been selected towards having shorter 3'UTRs, probably to avoid miRNA regulation (86). An increase in mRNA isoforms with short 3'UTRs has also been reported in proliferating cells, a strategy that might be adopted by cancer cells (87). Target sites within 5'UTRs, open reading frames (ORFs) and promoter regions have also been reported (80,88,89), but are generally less effective than those within the 3'UTR (85).

MiRNAs mediate regulation of mRNA through destabilization of the target followed by translational repression or degradation, or a combination of the two. Regardless of the mechanism the ultimate result is reduced protein output (90). Translational repression can occur in the absence of mRNA degradation, but the effects on mRNA and protein levels are generally modest compared to target degradation, which account for most of the miRNA-mediated repression (91,92). Target destabilization is initiated by TNRC6 (GW182-related protein) in the miRISC through recruitment of poly(A)-binding proteins (PABP) and deadenylase complexes (93,94). Several mechanisms for translational repression have been proposed, but the exact process is not fully understood. However, a general consensus is that components of the miRISC interfere with the mRNA 5'cap to block translation initiation (95,96). Deadenylation of mRNA makes it unstable and can either lead to progressive 3'-5' decay by exonucleases, or further recruitment of decapping complexes that eventually leads to 5'-3' exonucleolytic degradation (Reviewed in (97)). Although repression is the primary mechanism of miRNA-mediated mRNA regulation, other types of regulation of have also been described, such as translational activation and heterochromatin formation (98,99).

1.3 MicroRNA in cancer

The first indication that miRNAs could have a potential role in cancer came from the very first miRNAs discovered, the *C.elegans* lin-4 and let-7. Inactivation of lin-4 and let-7 caused specific epithelial cells to undergo additional cell divisions instead of their normal differentiation, thereby suggesting a role of miRNAs in abnormal cellular processes (54,55). Indeed, altered miRNA expression has been observed in various types of cancers, consisting of both up – and downregulated miRNAs (67,100-103). The discovery that miRNAs are involved in regulation of oncogenes and tumor suppressor genes has strengthened the theory that miRNAs play a central role in the underlying mechanisms of human cancer (104-107).

Differential expression of miRNAs between normal and tumor specimens has been described as a result of deregulation of key proteins in the miRNA biogenesis pathway (Drosha-DGCR8, Dicer1, AGO2 and GW182), epigenetic changes, and the location of a significant number (>50%) of miRNA genes in regions of genetic instability, known as fragile sites (108-112). However, mechanisms leading to deregulated miRNA expression in cancer are not fully understood and research in this area is lacking (113).

1.3.1 MicroRNA in colorectal cancer

MiR-143 and miR-145 were the first miRNAs associated with CRC. Michael and colleagues observed a significant downregulation of miR-143 and miR-145 in CRC compared to normal tissue (114), which was later shown to elicit tumor suppressor activity (115). Since then, a range of aberrantly expressed miRNAs have been associated with development and progression of CRC (116-119), suggesting that miRNAs could serve as novel biomarkers for early detection, prognosis and treatment response in CRC patients.

MicroRNAs as early detection biomarkers

As previously mentioned, early detection is key to improved survival in CRC patients and the search for early detection biomarkers have gained much attention in recent years. Simple methods for extraction and stability of miRNA under different conditions in body fluids, feces, and tissue make them ideal candidates as non-invasive biomarkers to improve early detection of CRC (120,121). Ng and colleagues presented the first evidence that miRNAs could be used for early detection of CRC by successfully discriminating CRC patients from control subjects, based on overexpression of miR-92 in plasma (122). Similar findings were reported in a study by Huang et al. (123). MiR-135 and miR-135b have been proposed as early detection biomarkers because they were shown to target APC in human CRC cells (124). APC mutations occur early in CRC genesis, thereby implicating overexpression of miR-135 as an early event in CRC that might be exploited in early detection. In a recent study, a progressive increase in miR-135b in normal colonic tissue to adenomas and CRC was reported, and higher levels of miR-135b were detected in tissue with loss of APC (125). Furthermore, elevated expression of miR-135 and members of the miR-17-92 cluster in colonocytes isolated from feces has been detected in patients with adenomas and CRC compared to healthy controls (126). Other promising miRNAs for early detection include miR-21, miR-31, miR-29a, and other members of the miR-17-92 cluster (127). In addition, miRNA has been detected in circulating cell-derived exosomes (128). Exosomes are found in most bodily fluids and several studies have reported that miRNAs retained in exosomes are similar to those of their originating cancer cells (129-131), suggesting that exosomal miRNA could have potential value in cancer diagnostics.

Initial studies regarding miRNA as biomarkers mainly concentrated on single or only a few miRNAs. However, a range of miRNAs are aberrantly expressed in CRC patients compared to healthy individuals, indicating that miRNA profiles might be better suited for detection of

CRC and also for discovery of novel biomarkers. Indeed, several studies have used miRNA expression profiling in the search for minimally invasive biomarkers in CRC (132-136), as well as in other human cancers and diseases with promising results (137-140).

Prognostic and predictive value of microRNA

Biomarkers that are able to predict treatment response, recurrence and clinical outcome are highly desired to improve survival and optimize treatment of CRC patients. Many studies have demonstrated the utility of miRNAs as prognostic and predictive biomarkers, but inconsistent results make it difficult to draw any definite conclusions.

Studies on the prognostic value of miRNAs have demonstrated an association between miRNA and survival of CRC patients. MiR-21 is a highly relevant miRNA in CRC and upregulation of miR-21 have been related to decreased overall survival and disease-free survival (141). However, several studies have failed to detect any significant correlation between miR-21 and prognostic factors and instead identified other miRNAs, such as overexpression of miR- 200c, miR-185, miR-221 and miR-182, and downregulation of miR-133b, miR-150 and miR-378, that correlated with poor survival rates (142-147). Several miRNAs have also been associated with shorter disease-free survival (DFS), including elevated levels of miR-183, miR-17-3p and miR-106a, and low levels of miR-15a and miR-16 (148-150).

In addition to survival rates, clinicopathological factors are important predictors of prognosis in CRC. Hur and colleagues were able to identify a panel of 3 miRNAs (miR-10b, miR-885-5p and let-7i) specific for mCRC patients (151). MiR-200c has also been related to CRC progression and metastases (152). In a study by Chen et al., they showed that levels of miR-200c and miR-210 were significantly elevated in patients that developed metastases or experienced recurrence after treatment compared to those that did not (153), indicating miR-200c and miR-210 as potential biomarkers to predict metastasis and local recurrence in CRC. MiR-155 is another miRNA found to be associated with metastases and also lymph node metastases and advanced TNM stage (154,155). Upregulation of miR-31 has been found to correlate with increased TNM stage (115,116), but results are varied and in a study by Slaby et al., no association with clinical or pathological staging was reported. However, they found that low expression of miR-31 correlated with poor tumor differentiation grade (156). Other studies have identified differentially expressed miRNAs that could distinguish between

patients with different TNM stages (157,158), and miRNA expression has also been used to discriminate MSI from MSS (microsatellite stability) tumors as well as colon cancer from rectal cancer (159,160).

The ability to predict a therapeutic response to a certain therapeutic agent is an important application for miRNA in CRC. Drug response varies between patients and use of miRNAs to predict the effect of chemotherapy allows for a more personalized approach to the management of CRC, as well as it would prevent overtreatment and toxic side effects for those who otherwise would have no benefit and provide better outcome for those who would have an effect. Several miRNAs have been associated with increased resistance to chemotherapy, such as miR-21, miR-320e and miR-155, whereas others have shown to increase chemosensitivity, including miR-150 and miR-129 (144,161). In a study by Kheirelseid et al., they identified a miRNA expression signature (miR-16, miR-590-5p and miR-153) that could predict complete versus incomplete response to neoadjuvant chemoradiotherapy in rectal cancer, and two miRNAs (miR-519c-3p and miR-561) that predicted good versus poor response (162).

It is widely known that patients with mutated KRAS have poor response to anti-EGFR therapy, and recent studies have identified certain miRNAs that are able to predict response to this treatment modality. For example, upregulation of miR-31-3p and miR-31-5p in mCRC patients positive for KRAS mutation have been associated with reduced response to anti-EGFR therapy. Furthermore, miR-31-3p and -5p are significantly overexpressed in patients with poor response to cetuximab (anti-EGFR drug) compared to responders, indicating that miR-31 could be used to distinguish patients with different response to anti-EGFR therapy. A signature of three clustered miRNAs (miR-99a, let-7c and miR-125b) have been reported to identify mCRC patients with wt KRAS that have better prognosis when treated with anti-EGFR therapy (163).

In summary, several miRNAs have emerged as promising biomarkers for early detection, prognosis and clinical management of CRC. However, challenges still remain to successfully translate the knowledge on miRNA into a clinical routine setting. Interpretation of the immense miRNA expression data that have been generated the past years has proven to be difficult, and further validation of the expression of miRNAs is required to assess the potential clinical value of the aforementioned candidates.

2. Aims of the study

The main aim of the current study was to identify microRNA in serum of CRC patients by small RNA sequencing and investigate the correlation between microRNA expression levels and clinical and histopathological characteristic of the patients, to search for non-invasive biomarkers in CRC.

The second aim of the study was to investigate the effect of aberrantly expressed microRNAs in CRC tissue, found by Mjelle et al. (unpublished work), on CRC cell growth.

3. Materials and methods

3.1 Materials

Materials used in this study are listed in (Appendix table B.1.1.)

3.2 Patient samples

Patients included in this study were initially recruited from two Norwegian hospitals (St.Olavs Hospital and Hamar Hospital) between January 2006 and June 2008. All patients (n = 562) were newly diagnosed with CRC. Tumor specimens were obtained from patients that underwent surgery or had a biopsy, and blood samples were collected either before or after treatment. Patient samples were stored at -80°C in a research Biobank after collection. Molecular tests were performed on paraffin-embedded tumor specimens from the patients, including MSI markers, mutation of the BRAF oncogene (V600E), and methylation analyses of MMR genes associated with Lynch Syndrome. KRAS mutation analysis was carried out later. Written informed consent was obtained from each patient, and the study was approved by The Regional Committee for Ethics in medical research and The National Data Inspectorate (164,165).

In the initial cohort of CRC patients, 132 were diagnosed with rectal and rectosigmoid cancer. From the patients diagnosed at St.Olavs Hospital (n=102), 96 patients were randomly included in this study. Relevant data were collected from the patients' medical records and data was also obtained from The Norwegian Cancer Registry.

3.3 RNA isolation

Total RNA were isolated from 200µl patient serum (four samples 250µl) using the QIAGEN miRNeasy serum/plasma kit. In brief, QIAzol lysis buffer (1000µl) were added to the sample to stabilize the RNA by eliminating ribonucleases, cellular DNA and proteins released by cell lysis. Addition of chloroform (200µl) and subsequent centrifugation allowed phase separation of the lysate, and the upper aqueous supernatant was separated and mixed with ethanol (2:1 ratio to volume of supernatant) before loaded onto the membrane in the spin columns provided in the kit. RNA bound to the column and contaminants were washed away before RNA was eluted using RNase-free water. Isolated RNA was stored at -80 °C. The miRNeasy

serum/plasma internal Spike-in control (C.elegans miR-39 miRNA mimic) was not used in this experiment.

RNA quantification and quality assessment of isolated RNA

Isolated RNA was measured using NanoDrop ™ ND-1000 spectrophotometer to give an indication on RNA purity and concentration. For further assessment of RNA quality and relative size, a few randomly selected samples was measured using Eukaryote total RNA pico assay on the 2100 Bioanalyzer. Results showed that small RNAs were present in the samples at acceptable concentrations to continue the library preparation (Example in Appendix Fig. B.2.1). It was assumed that the results of these samples were representative for all RNA samples. For total RNA assays, a ribosomal RNA ratio is determined giving an indication on RNA integrity. Ribosomal RNA (rRNA) is not expected to be present in cell-free serum, so the typical rRNA (ribosomal RNA) 28S:18S ratio and RNA integrity number (RIN) were not applicable.

3.4 Preparation of cDNA library for small RNA sequencing

Small RNA sample preparation was performed using NEBNext® Multiplex Small RNA Library prep set for Illumina (Set 1) according to the manufacturer's instructions. Briefly, 3' and 5' adaptors were sequentially ligated to serum total RNA, using 6µl input RNA per sample. A mix of ten different calibrator oligoribonucleotides (0.25µl) with known sequence and concentration were added in the 3'ligation step and used as internal standards as described by Hafner and colleagues (166). Calibrator sequences are listed in (Appendix table B.1.2) The following steps included reverse transcription of the ligated fragments, amplification by PCR for 13 cycles using Index primers from NEBNext® Multiplex Small RNA Library prep set for Illumina Set 1 and Set 2, and gel purification. The miRNA fragments were sequenced on the Illumina HiSeq system using 50 base pair single read, at the Genomics Core Facility (GCF) in Trondheim.

Quality control of cDNA library

Quality controls of the cDNA libraries were performed using High Sensitivity DNA assay on 2100 Bioanalyzer. Results showed that cDNA library construction was successful (Example in Appendix Fig. B.2.2).

3.5 Processing of sequence data

Quality control check of the raw sequence data was performed using fastQC (167). Trimming of sequence adapters from the 3'end of the raw sequences was performed using cutadapt-1.2.1 (168). The cut sequences were collapsed with the fastx_collapser tool into single unique reads along with their total read count and mapped to the human (hg38) genome using bowtie2, allowing for up to 10 alignments per read to account for reads from duplicated miRNA loci (bowtie2 – k10). Reads overlapping with mature miRNA loci were identified using htseq-count from the HTseq python package (169). These reads were further filtered to identify those with perfect alignment to the genome, and the total read count for mature miRNAs were then computed by summing the total read count per sequence (isomiR) overlapping each miRNA locus. Mature miRNAs and non-coding RNAs were annotated using miRBase (Release 21, 2014) and Rfam (v11), respectively. IsomiR variants were detected using SeqBuster (170) combined with a panel of in-house perl and R-scripts, available upon request. IsomiRs with mismatches to the genome were discarded from the analysis, as these could not be excluded as sequencing errors. However, isomiRs with non-templated addition at the 3'end were included in the analysis. Differentially expressed miRNAs and isomiRs were identified using the Bioconductor package limma combined with the voom transform (171,172). All miRNA sequence information was retrieved from miRBase (173). In order to compare miRNA expression between samples, read counts were normalized using the spike-in normalization factors calculated in limma, followed by reads per million (rpm) normalization.

3.6 Functional assay in cultured cells

Cancer cell line

The human SW480 colon cancer cell line were cultured as adherent cells in 75cm^3 dishes using Leibowitz L-15 medium with L-glutamine, supplemented with 10% fetal bovine serum (FBS) and humidified in 5% CO_2 at 37 °C. The cells were passaged at ~80 confluency by washing twice with 10mL pre-heated Phosphate Buffered Saline (PBS), followed by 1mL Trypsin-EDTA. Cells were split at a 1:10 ratio.

Selecting miRNA mimics and inhibitors for transfection

The CRC research group has previously shown that several miRNAs are differentially expressed between tumor tissue and adjacent normal mucosa in CRC (unpublished work, Mjelle et al). To verify these findings we wanted to test if the same miRNAs that were differentially expressed in CRC tissue could alter the growth of SW480 CRC cells when overexpressed or inhibited. The hypothesis was that miRNA overexpressed in tumor tissue represent oncomiRs that stimulate cell growth and that miRNAs under-expressed represent tumor suppressor miRNAs that protects against aberrant growth. By adjusting their normal levels by inhibitors and mimics we expected to see reduced cell growth compared to controls. 18 miRNA inhibitors and 33 miRNA mimics were selected. MiRNA inhibitors and mimics are listed in (Appendix table B.1.3)

Cell transfection

SW480 cells were seeded in 96-well plates (~1 x 10^3 cells) prior to transfection. For each well, 0.6µL miRNA mimic or miRNA inhibitor or negative controls were diluted in 7.5µL Opti-MEM medium. Transfection was performed using Lipofectamine (0.15µL) in Opti-MEM (7.5µL). The transfection complex were added to the cells (10µL per well for miRNA mimics and 20µL for miRNA ihibitors, and corresponding amount of negative control) and incubated at 37 °C in a CO_2 incubator for 24h before functional analysis.

Cell Viability Assay

Cell Viability was measured using the resazurin-based PresoBlue Assay. After 24, 48, 72 and 96 hours of transfection 10µL PrestoBlue reagent were added to each well and incubated for 1 hour at 37°C in 5% CO_2. Viable cell numbers were evaluated by measuring fluorescence intensity. All experiments were performed in triplicates.

3.7 Statistical analysis

Kaplan-Meier and Cox proportional hazard regression models were used to assess the influence of miRNA levels on overall survival, measured from the date of diagnosis. The Cox regression model was run with coxph package and *p*-values were adjusted for multiple testing using Benjamin Hochberg correction. Student's t-test and linear regression were used to identify miRNAs associated with clinicopathological parameters. When linear regression was

used, continuous clinical parameters were converted to log2. In the transfection experiment, *P*-values were calculated using a two-tailed t-test by comparing the replicates measured at time point 96h. All statistical analyses were performed using the R software (v3.2.2). *P*-values <0.05 were considered statistically significant.

4. Results

4.1 Patient characteristics

General information and relevant data collected from the patient's medical records used in the results are presented below (Table 4.1). Assessment of distant metastases, local recurrence, and tumor classification according to the 5th edition of the TNM staging system, was made with help from an experienced oncologist and a surgeon (5th edition TNM staging system and stage groups are listed in Appendix tables B.3.1 and B.3.2) respectively.

Table 4.1. Clinical and histopathological characteristics of the investigated patient cohort.

Gender	Men	53 (55,2%)
	Women	43 (44,8%)
Age at diagnosis	<39	3
	40-49	5
	50-59	19
	60-69	32
	70-79	19
	80-89	17
	>90	1
Mean age at diagnosis: 65,7 (34-94)		
T	Tis	1
	T1	5
	T2	11
	T3	43
	T4	36
N*	N0	57
	N1	14
	N2	23
M	M0	75
	M1	21
Stage Grouping	0	1
	I	13
	II	38
	III	23
	IV	21
KRAS mutation**	Wild type	70 (73%)
	Mutation	26 (27%)
Tumor differentiation grade***	High	3
	Moderate	69
	Moderate - low	3
	Low	6
CEA****	<5	50

	5 - 10	11
	10 - 100	17
	>100	3
Serum collection***	Before treatment	53
	After treatment	43

* N-stage of two patients could not be assessed

** Different KRAS mutations were tested in the patients; G12D (n=16), G13D (n=6), G12A (n=3) and G125 (n=1)

*** For 15 patients, tumor differentiation grade was not listed in the medical records

**** Results from CEA measurement was either unavailable from medical records or analysis was not conducted before preoperative treatment was given, for 15 patients.

***** Serum was collected before treatment or after treatment. In the after-treatment group (n=43), serum was either collected after preoperative treatment alone (n=34), after preoperative treatment and surgery (n=5), or after surgery alone (n=3).

4.2 Sequencing statistics

In average, 10 964 086 reads mapping to the human genome were obtained per sample. Multidimensional scaling (MDS) of the mature miRNAs revealed no clear subgrouping of the samples (Supplementary Fig. A.1.A) and read counts were relatively evenly distributed for mature miRNAs across all samples (Supplementary Fig. A.1.B). Sequencing libraries were dominated by small cytoplasmic RNAs (scRNAs), miRNAs and long non-coding RNAs (lncRNAs) (Supplementary Fig. A.1.D). A small fraction of rRNA, tRNA, small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA) were also detected. A total of 498 mature miRNAs were detected in the dataset. 418 miRNAs were expressed in 50% of the samples and 414 miRNAs were expressed with cpm>1 in 50% of the samples. The most abundantly expressed miRNA was the blood-specific miRNA miR-486-5p with an average expression of 175 660 reads, followed by miR-423-5p with an average expression of 97 157 reads. An overview of the 20 most abundant miRNAs are shown in (Supplementary Fig. A.2.) 8757 isomiRs (sequence variants of mature miRNA sequences) were detected across the 96 serum samples. The isomiRs were divided in six groups according to common nomenclature. IsomiRs with 3'tailing and trimming as well as non-template additions (NTAs) were the most common types (Supplementary Fig. A.3.A-B). IsomiRs containing mismatches to the genome were removed, resulting in 3758 isomiRs to be used for downstream analysis. Of these, 2769 isomiRs were expressed in more than 50% of the samples and 1196 isomiRs were expressed

with cpm>1 in 50% of the samples. The miRNA miR-486-5p had 105 unique isomiRs and 111 miRNAs had more than 10 unique isomiRs.

In total, 28 unique spike-in sequences were detected in the sequencing data mapping to eight different calibrators (Supplementary Fig A.1.E). Calibrator 10 had the highest expression with an average of 10415 reads. No sample clustering of the calibrators were observed, indicating that they were evenly distributed across samples during the library preparation. From this we conclude that the sample preparation and sequencing were performed without any significant bias across samples.

4.3 Association between microRNA expression and patient survival

Survival analysis was performed to investigate the potential prognostic value of circulating miRNA levels in CRC patients. Overall survival across all patients relative to TNM stage (I-II, III and IV) is illustrated as a Kaplan-Meier survival curve in (Fig. 4.1), showing that increasing TNM stage confers worse survival. High expression levels of four mature miRNAs (miR-320a, miR-320b, miR-320c, miR-320d) was found to be significantly associated with poor overall survival (p<0.05) compared to low levels of these miRNAs. In addition, high levels of 15 isomiRs and low levels of four isomiRs had a significant negative influence on OS (p<0.05). Separate Kaplan-Meier survival curves for mature miRNAs and isomiRs are presented in (Fig. 4.2) and hazard ratios are listed in (Table 4.2 and Table 4.3), for mature miRNAs and isomiRs respectively.

Figure 4.1

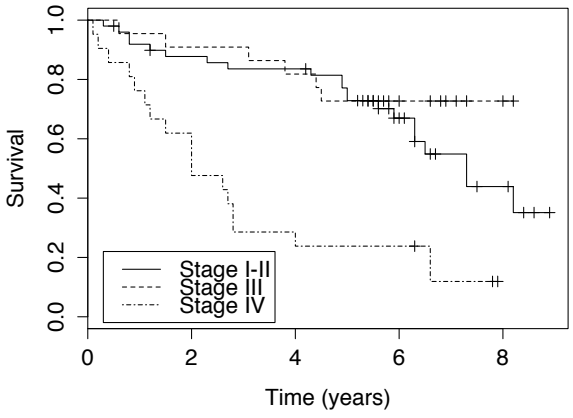
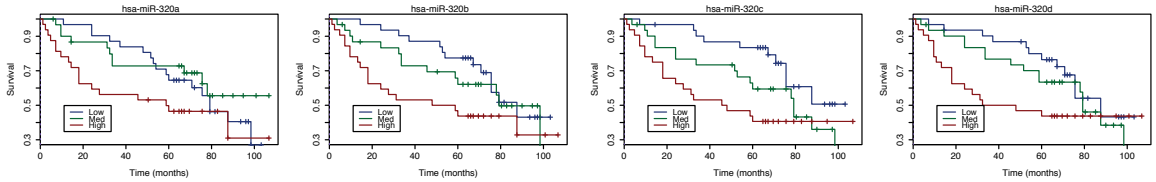


Figure. 4.1. **Increasing TNM stage is associated with poor survival (OS) in rectal cancer patients, measured from date of diagnosis**. Illustrated as a Kaplan-Meier survival curve.

Figure 4.2

A


hsa-miR-320a
hsa-miR-320b
hsa-miR-320c
hsa-miR-320d
Survival
Time (months)
Low
Med
High

B

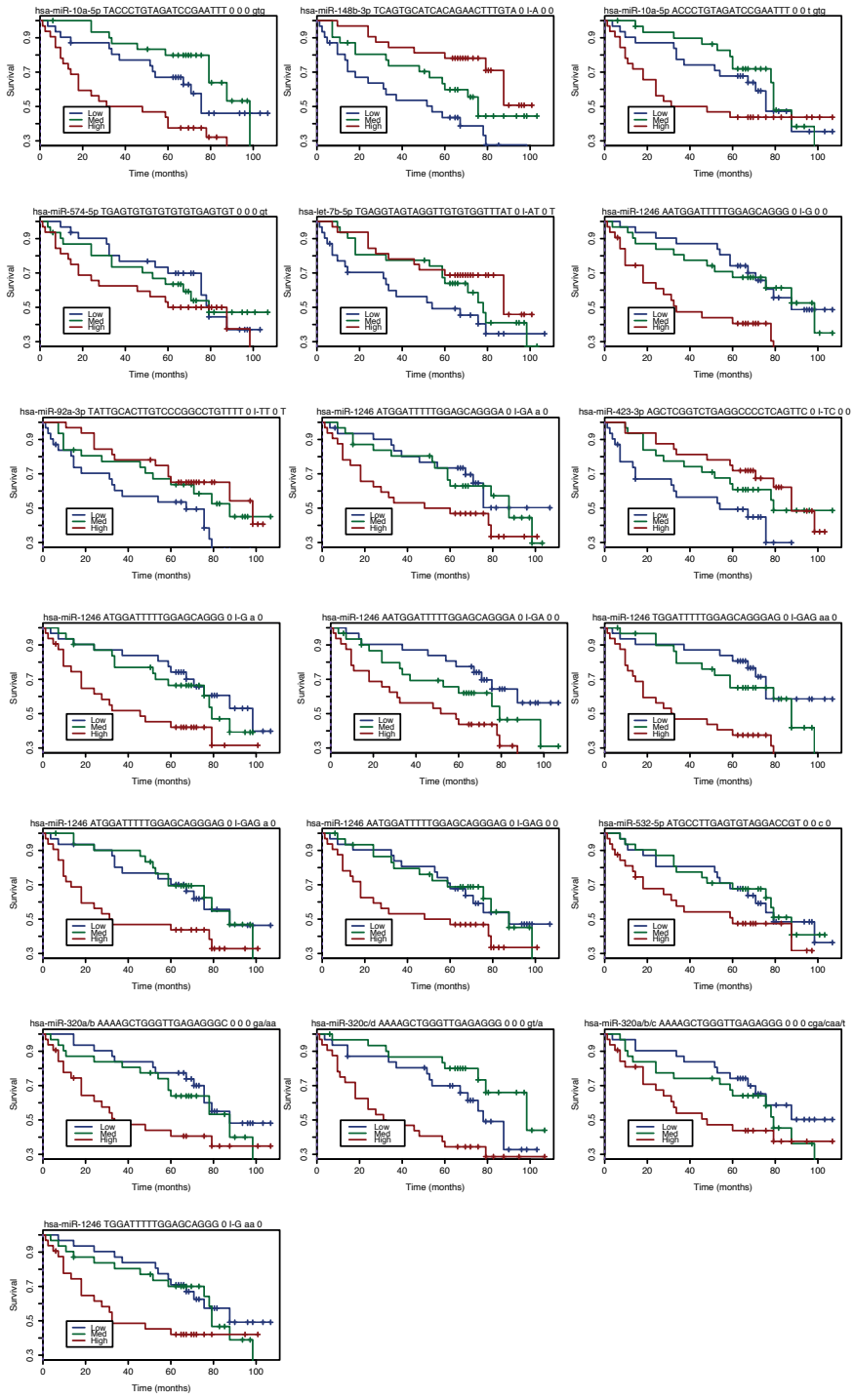

hsa-miR-10a-5p TACCCTGTAGATCCGAATTT 0 0 0 gtg
hsa-miR-148b-3p TCAGTGCATCACAGAACTTTGTA 0 I-A 0 0
hsa-miR-10a-5p ACCCTGTAGATCCGAATTT 0 0 t gtg
hsa-miR-574-5p TGAGTGTGTGTGTGTGAGTGT 0 0 0 gt
hsa-let-7b-5p TGAGGTAGTAGGTTGTGTGGTTTAT 0 I-AT 0 T
hsa-miR-1246 AATGGATTTTTGGAGCAGGG 0 I-G 0 0
hsa-miR-92a-3p TATTGCACTTGTCCCGGCCTGTTTT 0 I-TT 0 T
hsa-miR-1246 ATGGATTTTTGGAGCAGGGA 0 I-GA a 0
hsa-miR-423-3p AGCTCGGTCTGAGGCCCCTCAGTTC 0 I-TC 0 0
hsa-miR-1246 ATGGATTTTTGGAGCAGGG 0 I-G a 0
hsa-miR-1246 AATGGATTTTTGGAGCAGGGA 0 I-GA 0 0
hsa-miR-1246 TGGATTTTTGGAGCAGGGAG 0 I-GAG aa 0
hsa-miR-1246 ATGGATTTTTGGAGCAGGGAG 0 I-GAG a 0
hsa-miR-1246 AATGGATTTTTGGAGCAGGGAG 0 I-GAG 0 0
hsa-miR-532-5p ATGCCTTGAGTGTAGGACCGT 0 0 c 0
hsa-miR-320a/b AAAAGCTGGGTTGAGAGGGC 0 0 0 ga/aa
hsa-miR-320c/d AAAAGCTGGGTTGAGAGGG 0 0 0 gt/a
hsa-miR-320a/b/c AAAAGCTGGGTTGAGAGGG 0 0 0 cga/caa/t
hsa-miR-1246 TGGATTTTTGGAGCAGGG 0 I-G aa 0
Survival
Time (months)
Low
Med
High

Figure 4.2. MicroRNA expression levels affect overall survival in rectal cancer patients. A) High expression levels of four mature miRNAs (miR-320a, miR-320b, miR-320c and miR-320d) were associated with poor survival of rectal cancer patients. B) High expression levels of 15 isomiRs and low expression levels of four isomiRs had negative influence on overall survival. Some of the isomiRs of the miR-320 family could not be uniquely annotated to either family member and were therefore given a combined name. Explanation of the names of the isomiRs are given in (Table 4.3).

Table 4.2. Multivariate analysis for overall survival (mature miRNA)

MicroRNA	HR*	95% CI	*P*-value	Adjusted *p*-value
miR-320d	1.61	1.34 - 1.95	6.71e-07	0.0001
miR-320c	1.64	1.33 - 2.02	3.89e-06	0.0004
miR-320b	1.63	1.29 - 2.04	2.31e-05	0.0017
miR-320a	1.73	1.30 - 2.34	0.0002	0.0099

Table 4.3. Multivariate analysis for overall survival (isomiRs)

MiRNA	Sequence	Mismatch, NTA, 5'modification, 3'modification **	HR ***	95% CI	*P* value	Adjusted *P* value
miR-320c/d*	AAAAGCTGGGTTGAGAGG	0,0,0,gt/a	1.49	1.27 - 1.77	0.000003	0.0013
miR-320a/b/c	AAAAGCTGGGTTGAGAGGG	0,0,0,cga/caa/t	1.67	1.33 - 2.11	0.00001	0.0020
miR-1246	TGGATTTTTGGAGCAGGG	0,I-G,aa,0	1.45	1.23 - 1.73	0.00002	0.0025
miR-1246	TGGATTTTTGGAGCAGGGAG	0,I-GAG,aa,0	1.44	1.22 - 1.69	0.00002	0.0025
miR-1246	ATGGATTTTTGGAGCAGGG	0,I-G,a,0	1.47	1.23 - 1.76	0.00002	0.0025
miR-1246	ATGGATTTTTGGAGCAGGGA	0,I-GA,a,0	1.42	1.21 - 1.66	0.00002	0.0025
miR-1246	AATGGATTTTTGGAGCAGGGA	0,I-GA,0,0	1.41	1.19 - 1.65	0.00003	0.0028
miR-1246	AATGGATTTTTGGAGCAGGG	0,I-G,0,0	1.48	1.23 - 1.78	0.00003	0.0028
miR-320a/b	AAAAGCTGGGTTGAGAGGGC	0,0,0,ga/a	1.56	1.25 - 1.96	0.00009	0.0069
miR-1246	AATGGATTTTTGGAGCAGGGAG	0,I-GAG,0,0	1.42	1.19 - 1.69	0.00009	0.0069
miR-148b-3p	TCAGTGCATCACAGAACTTTGTA	0,I-A,0,0	0.64	0.51 - 0.82	0.0003	0.0195
miR-10a-5p	TACCCTGTAGATCCGAATTT	0,0,0,gtg	1.57	1.23 - 2.01	0.0004	0.0224
miR-1246	ATGGATTTTTGGAGCAGGGAG	0,I-GAG,a,0	1.39	1.16 - 1.69	0.0005	0.0298
let-7b-5p	TGAGGTAGTAGGTTGTGTGGTTTAT	0,I-AT,0,T	0.59	0.44 - 0.80	0.0006	0.0330
miR-92a-3p	TATTGCACTTGTCCCGGCCTGTTTT	0, -TT,0,T	0.65	0.51 - 0.83	0.0006	0.0330
miR-423-3p	AGCTCGGTCTGAGGCCCCTCAGTTC	0,I-TC,0,0	0.65	0.51 - 0.84	0.0007	0.0359
miR-532-5p	ATGCCTTGAGTGTAGGACCGT	0,0,c,0	1.96	1.32 - 2.91	0.0009	0.0424
miR-10a-5p	ACCCTGTAGATCCGAATTT	0,0,t,gtg	1.38	1.14 - 1.67	0.0009	0.0431
miR-574-5p	TGAGTGTGTGTGTGTGAGTGT	0,0,0,gt	2.11	1.34 - 3.29	0.0012	0.0495

* IsomiRs of the miR-320 family were given combined names because the isomiRs could not be uniquely annotated to either family member.

** NTA (non-templated nucleotide addition): Added nucleotides are indicated after "I". 3'/5' modifications: Lower case letters indicate trimming and upper case letters indicate tailing with respect to canonical sequence.

***HR: hazard ratio. The hazard ratio describes to what extent miRNA expression influence the duration of the disease (CRC). HR=1, no influence. HR > or < 1, greater or less influence.

4.4 Association of microRNA expression levels with clinicopathological parameters

Associations between miRNA expression levels and clinicopathological parameters were also investigated, using Students t-test and linear regression. Variables included in the analysis were TNM stage groups (stage I-II, III and IV), preoperative treatment, development of metastasis in non-metastatic patients after diagnosis, tumor differentiation grade, local recurrence and clinical routine analyses (CEA, hemoglobin, leukocytes, thrombocytes, albumin, LD and CRP). Mature miRNAs and sequence variants (isomiRs, see Supplementary Fig. A.3 for definition) associated with clinicopathological parameters are listed in (Table 4.4 and 4.5), respectively. $P<0.05$ for all miRNAs.

Serum was collected either before or after some form of treatment in the patients included in this study. In patients that had received treatment (n=43), serum was either collected after preoperative treatment alone (n=34), after preoperative treatment and surgery (n=5), or after surgery alone (n=3). We sought to identify miRNAs differentially expressed between patients that received preoperative treatment alone prior to serum collection and patients that did not receive preoperative treatment before serum was collected. From the results, three mature miRNAs (miR-10b-5p, miR-150-5p and miR-215-5p) showed significant differential expression between the before- and after-treatment groups. Results also revealed changed expression of sequence variants of miR-10b-5p and miR-150-5p and three additional variants of miR-125b-5p and miR-30a-5p between the two groups (Fig. 4.3). Comparison of miRNA expression levels with the other clinicopathological parameters showed that 20 mature miRNAs and 47 isomiRs significantly associated with metastasis at diagnosis (stage IV) (Fig. 4.4). Of the 47 isomiRs, 31 were sequence variants of mature miRNAs and for 16 variants no mature sequence was detected. No significant association of miRNA expression levels with TNM stage I-III, non-metastatic patients that developed metastasis after diagnosis, tumor differentiation grade or local recurrence was found.

Table 4.4. Mature miRNAs associated with clinicopathological parameters

	MicroRNA	*log*FC	Average expression (*log*)	*P* value
Metastasis at diagnosis*	hsa-miR-320d	-2.03	7.36	6.8e-06
	hsa-miR-320c	-1.72	8.54	3.1e-05
	hsa-miR-10a-5p	-1.11	13.34	0.0001
	hsa-miR-320b	-1.43	10.01	0.0002
	hsa-miR-320e	-1.87	4.47	0.0002
	hsa-miR-1307-5p	-0.93	6.11	0.0014
	hsa-miR-4516	-1.19	4.95	0.0046
	hsa-miR-200c-3p	-1.05	5.89	0.0048
	hsa-miR-29a-3p	-0.91	8.44	0.0053
	hsa-miR-4488	-1.80	4.97	0.0053
	hsa-miR-4466	-1.97	2.00	0.0057
	hsa-miR-320a	-0.78	12.04	0.0068
	hsa-miR-296-3p	-2.08	1.62	0.0075
	hsa-miR-1246	-1.28	6.47	0.0143
	hsa-miR-200b-3p	-1.12	4.35	0.0144
	hsa-miR-200b-5p	-1.42	2.25	0.0159
	hsa-miR-200a-3p	-1.24	5.60	0.0194
	hsa-miR-3679-5p	-1.28	3.21	0.0476
	hsa-miR-4732-5p	0.58	5.77	0.0476
	hsa-miR-15a-5p	0.97	2.86	0.0500
Preoperative treatment	hsa-miR-150-5p	-0,99	8,39	0,0211
	hsa-miR-10b-5p	0,59	13,96	0,0437
	hsa-miR-215	0,84	6,52	0,0437
CEA	hsa-miR-320d	NA**	7,36	0,0015
	hsa-miR-320c	Na	8,52	0,0033
	hsa-miR-320e	NA	4,43	0,0033
	hsa-miR-320b	NA	9,99	0,0066
	hsa-miR-29a-3p	NA	8,43	0,0066
	hsa-miR-320a	NA	12,04	0,0113
	hsa-miR-29c-3p	NA	2,71	0,0259
	hsa-miR-200c-3p	NA	5,89	0,0446
Hb	hsa-miR-877-5p	NA	4,76	0,0313
	hsa-miR-451a	NA	14,26	0,0313

*Negative fold change indicates up-regulation in patients diagnosed with metastasis compared to non-metastatic patients at diagnosis.

** Not applicable (NA) because association between expression levels of isomiRs and CEA were performed using linear regression.

Table 4.5. IsomiRs associated with clinicopathological parameters.

Category/ MicroRNA	Sequence	NTA	5'modification	3'modification	*log*FC	Average expression (*log*)	*P* value
Metstasis at diagnosis*							
hsa-miR-320c/d	AAAAGCTGGGTTGAGAGG	0	0	gt/a	-2.37	6.14	6.6e-06
hsa-miR-10a-5p	ACCCTGTAGATCCGAATTT	0	t	gtg	-2-29	5.19	2.5e-05
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTT	0	0	gtg	-1.69	7.77	4.4e-05
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGT	0	0	g	-1.12	12.67	0,0003
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTG	0	0	tg	-1.19	8.84	0.0003
hsa-miR-320a/b/c	AAAAGCTGGGTTGAGAGGG	0	0	cga/caa/t	-1.42	7.29	0.0003
hsa-miR-1246	TGGATTTTTGGAGCAGGGAG	I-GAG	aa	0	-1.51	7.56	0.0003
hsa-miR-1246	ATGGATTTTTGGAGCAGGGA	I-GA	a	0	-1.76	6.49	0.0004
hsa-miR-10a-5p	ACCCTGTAGATCCGAATTTGT	0	t	g	-0.97	9.84	0.0006
hsa-miR-10a-5p	ATACCCTGTAGATCCGAATTTGT	0	A	g	-1.36	4.76	0.0009
hsa-miR-320a/b	AAAAGCTGGGTTGAGAGGGC	0	0	ga/aa	-1.32	8.99	0.0010
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGC	I-C	0	tg	-1.01	8.04	0.0016
hsa-miR-1246	ATGGATTTTTGGAGCAGGGAG	I-GAG	a	0	-1.37	7.39	0.0016
hsa-let-7d-3p	CTATACGACCTGCTGCCTTTC	0	0	t	-0.64	10.21	0.0039
hsa-miR-1246	TGGATTTTTGGAGCAGGG	I-G	aa	0	-1.29	7.89	0.0044
hsa-miR-1256	ATGGATTTTTGGAGCAGGG	I-GA	a	0	-1.26	7.52	0.0049
hsa-miR-10a-5p	ACCCTGTAGATCCGAATTTG	0	t	tg	-0.86	6.05	0.0085
hsa-miR-320a/b/c	AAAAGCTGGGTTGAGAGGGA	I-A	0	cga/caa/t	-1.19	4.62	0.0092
hsa-miR-1256	AATGGATTTTTGGAGCAGGGA	I-GA	0	0	-1.63	4.52	0.0092
hsa-miR-1307-5p	TCGACCGGACCTCGACCG	0	0	gct	-0.97	4.32	0.0092
hsa-miR-10a-5p	ACCCTGTAGATCCGAATTTGC	I-C	t	tg	-0.89	5.18	0.0094
hsa-miR-1256	AATGGATTTTTGGAGCAGGGAG	I-GAG	0	0	-1.32	6.28	0.0102
hsa-let-7i-5p	TGAGGTAGTAGTTTGTGCTA	I-A	0	gtt	0.68	4.08	0.0102
hsa-miR-320c	AAAAGCTGGGTTGAGAGGGT	0	0	0	-1.21	4.90	0.0117
hsa-let-7d-3p	TATACGACCTGCTGCCTTTC	0	c	t	-0.58	6.43	0.0117
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGA	I-A	0	tg	-0.72	6.62	0.0119
hsa-miR-532-5p	ATGCCTTGAGTGTAGGACCGT	0	c	0	-0.57	7.34	0.0124
hsa-miR-375	TTTGTTCGTTCGGCTCGCGT	0	0	ga	-1.65	6.41	0.0126
hsa-miR-143-3p	TGAGATGAAGCACTGTAGCA	I-A	0	tc	1.10	4.68	0.0126
hsa-miR-192-5p	CTGACCTATGAATTGACAGC	0	0	c	-0.78	7.29	0.0152
hsa-miR-486-5p	TCCTGTACTGAGCTGCCCCA	I-A	0	gag	0.61	10.03	0.0156
hsa-miR-200a-3p	TAACACTGTCTGGTAACGATG	0	0	t	-1.37	5.17	0.0187
hsa-miR-486-5p	TCCTGTACTGAGCTGCCCCT	I-T	0	gag	0.63	8.09	0.0206

hsa-miR-25-3p	CATTGCACTTGTCTCGGTCTGC	I-C	0	a	0.67	5.02	0.0214
hsa-miR-27b-3p	TTCACAGTGGCTAAGTTCT	0	0	gc	-0.59	8.86	0.0236
hsa-let-7b-5p	TGAGGTAGTAGGTTGTGTGGC	I-C	0	tt	0.61	9.49	0.0236
hsa-miR-95-3p	TTCAACGGGTATTTATTGAGC	0	0	a	-0.88	4.79	0.0303
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGTA	I-A	0	g	-0.69	4.88	0.0306
hsa-miR-25-3p	CATTGCACTTGTCTCGGTCTGAC	0	0	C	0.73	4.26	0.041
hsa-miR-574-5p	TGAGTGTGTGTGTGTGAGTGT	0	0	gt	-0.49	6.84	0.0462
hsa-miR-192-5p	TGACCTATGAATTGACAGC	0	c	c	-0.71	5.43	0.0497
Preoperative treatment							
hsa-miR-150-5p	TCTCCCAACCCTTGTACCAGT	0	g	0	-1.38	5.36	0.0007
hsa-miR-30a-5p	TGTAAACATCCTCGACTGG	0	0	aag	0.87	4.87	0.0117
hsa-miR-10b-5p	TACCCTGTAGAACCGAATTTG	0	0	tg	0.68	9.23	0.0183
hsa-miR-10b-5p	ACCCTGTAGAACCGAATTTGT	0	t	g	0.65	8.89	0.0238
hsa-miR-150-5p	TCTCCCAACCCTTGTACCAGTG	0	0	0	-0.92	7.72	0.0292
hsa-miR-10b-5p	TACCCTGTAGAACCGAATTTGT	0	0	g	0.62	13.54	0.0292
hsa-miR-125b-5p	TCCCTGAGACCCTAACTTGT	0	0	ga	0.75	7.43	0.0305
hsa-miR-10b-5p	ACCCTGTAGAACCGAATTTGC	I-C	t	tg	0.81	4.23	0.0453
hsa-miR-10b-5p	TACCCTGTAGAACCGAATTTGC	I-C	0	tg	0.63	8.90	0.0453
hsa-miR-30a-5p	TGTAAACATCCTCGACTGGAAG	0	0	0	0.63	7.19	0.0453
CEA							
hsa-miR-320c/d	AAAAGCTGGGTTGAGAGG	0	0	gt/a	NA**	6.14	0.0035
hsa-miR-320a	AAAGCTGGGTTGAGAGGGCG	0	a	a	NA	4.69	0.0178
hsa-miR-320a/b/c	AAAAGCTGGGTTGAGAGGG	0	0	cga/caa/t	NA	7.26	0.0343
hsa-miR-320a/b	AAAAGCTGGGTTGAGAGGGC	0	0	ga/aa	NA	8.99	0.0388
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTT	0	0	gtg	NA	7.75	0.0388
hsa-miR-139-5p	TCTACAGTGCACGTGTCTCCAG	0	0	t	NA	5.13	0.0478
Hb							
hsa-miR-451a	AAACCGTTACCATTACTGAG	0	0	tt	NA	8.58	0.0306

*Negative fold change indicates up-regulation in patients diagnosed with metastasis compared to non-metastatic patients at diagnosis.

** Not applicable (NA) because association between expression levels of isomiRs and CEA were performed using linear regression.

Figure 4.3

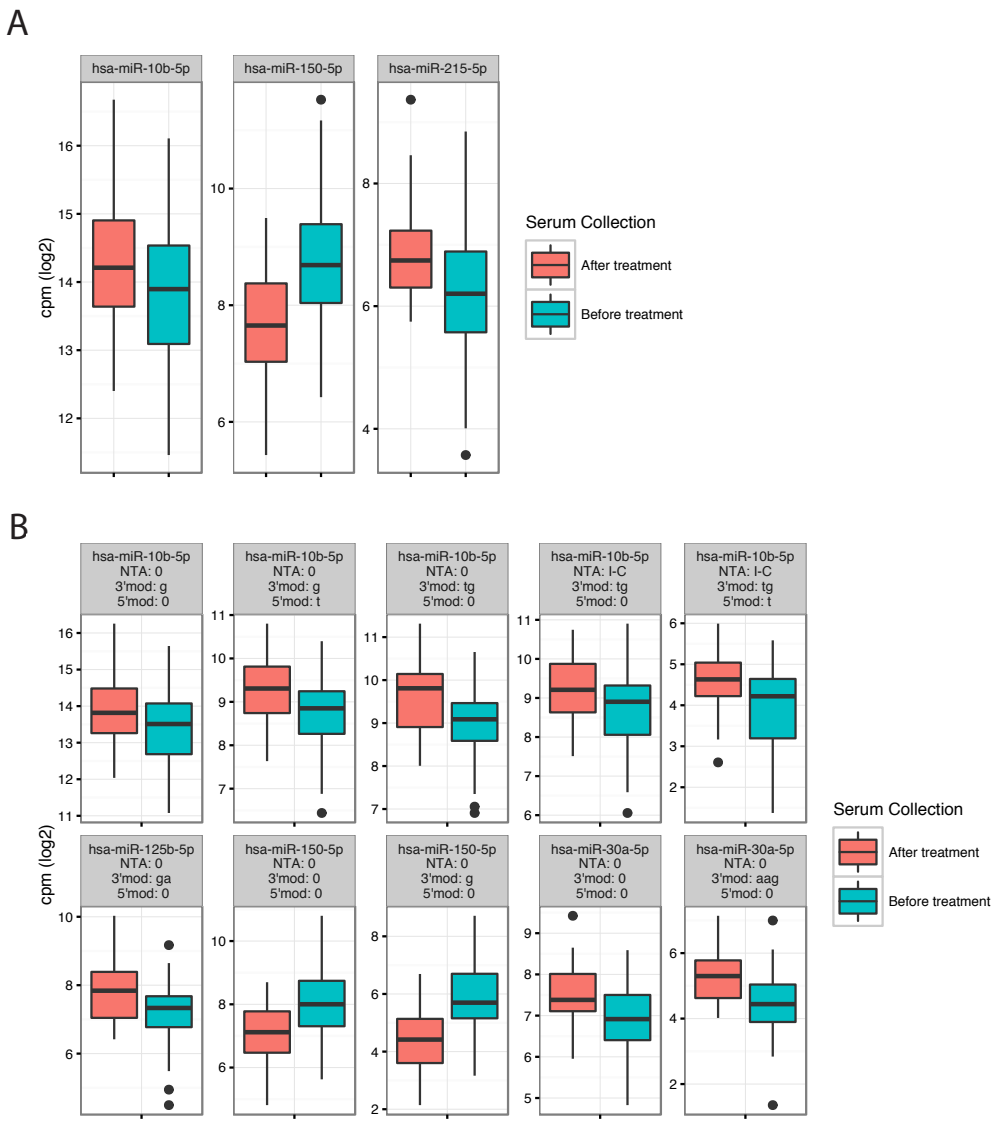


Figure 4.3. MicroRNAs show changed expression between rectal cancer patients that received preoperative treastment prior to serum collection and patients that did not receive treatment before serum collection. A) Levels of Mir-10b-5p and miR-215-5p were significantly higher and levels of miR-150-5p were significantly lower in serum of rectal cancer patients reveicing preoperative treatment compared to those who did not receive preoperative treatment (before serum collection). B) 10 isomiRs were differentially expressed in serum between the before-treatment and after-treatment patient groups.

Figure 4.4.A

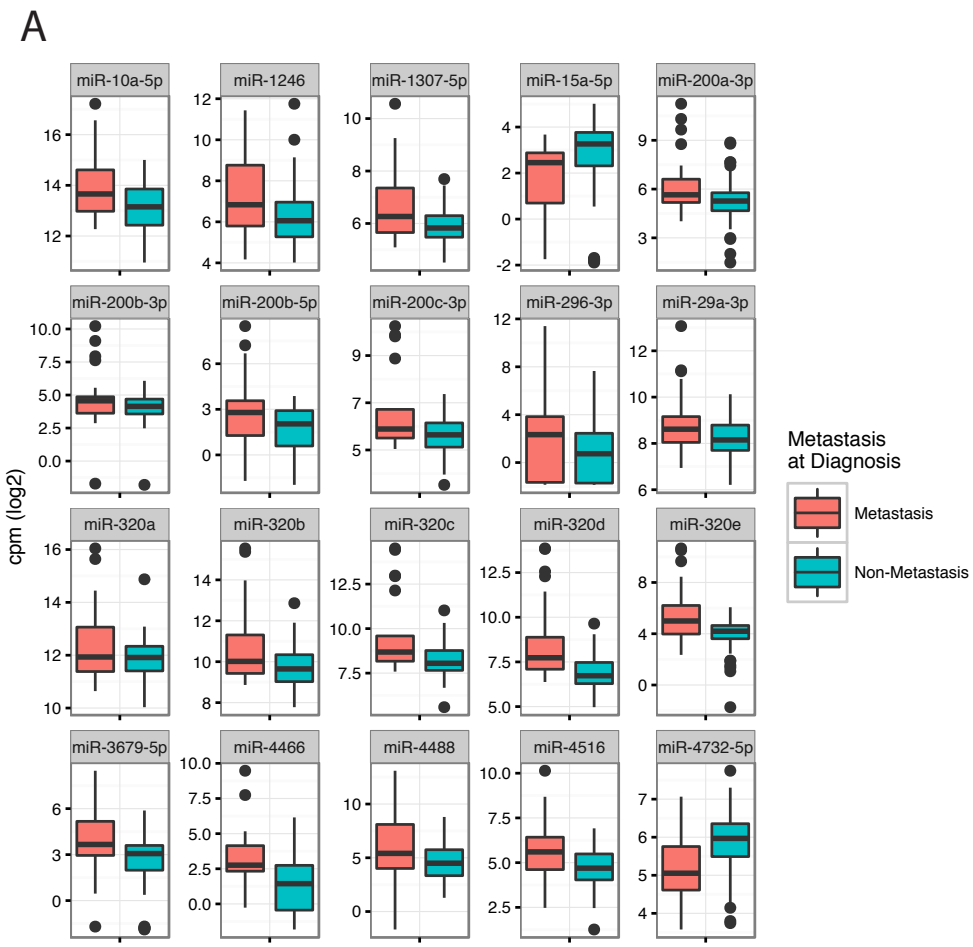

A
miR-10a-5p
miR-1246
miR-1307-5p
miR-15a-5p
miR-200a-3p
miR-200b-3p
miR-200b-5p
miR-200c-3p
miR-296-3p
miR-29a-3p
miR-320a
miR-320b
miR-320c
miR-320d
miR-320e
miR-3679-5p
miR-4466
miR-4488
miR-4516
miR-4732-5p
cpm (log2)
Metastasis at Diagnosis
Metastasis
Non-Metastasis

Figure 4.4.B

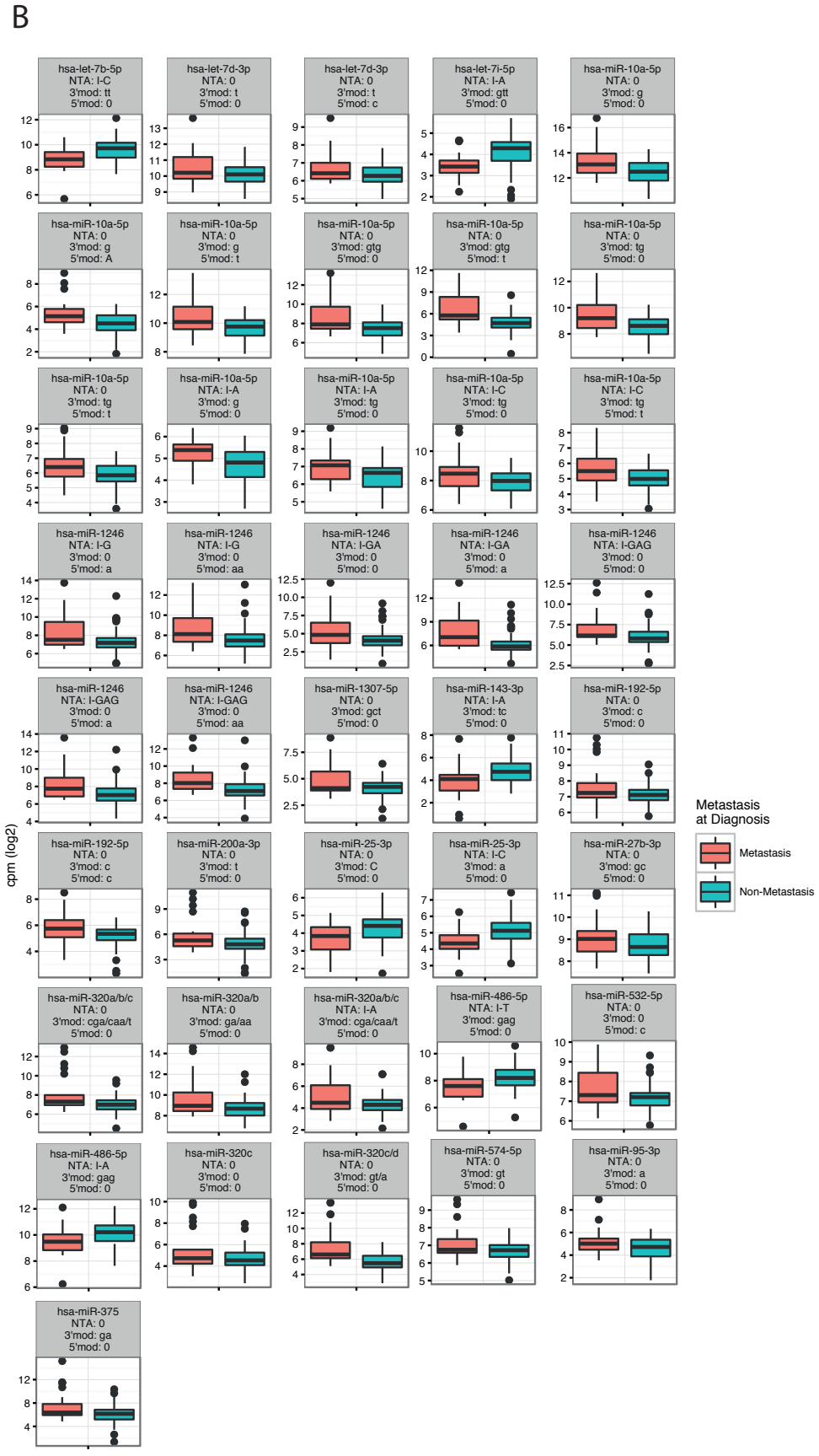

B
hsa-let-7b-5p NTA: I-C 3'mod: tt 5'mod: 0
hsa-let-7d-3p NTA: 0 3'mod: t 5'mod: 0
hsa-let-7d-3p NTA: 0 3'mod: t 5'mod: c
hsa-let-7i-5p NTA: I-A 3'mod: gtt 5'mod: 0
hsa-miR-10a-5p NTA: 0 3'mod: g 5'mod: 0
hsa-miR-10a-5p NTA: 0 3'mod: g 5'mod: A
hsa-miR-10a-5p NTA: 0 3'mod: g 5'mod: t
hsa-miR-10a-5p NTA: 0 3'mod: gtg 5'mod: 0
hsa-miR-10a-5p NTA: 0 3'mod: gtg 5'mod: t
hsa-miR-10a-5p NTA: 0 3'mod: tg 5'mod: 0
hsa-miR-10a-5p NTA: 0 3'mod: tg 5'mod: t
hsa-miR-10a-5p NTA: I-A 3'mod: g 5'mod: 0
hsa-miR-10a-5p NTA: I-A 3'mod: tg 5'mod: 0
hsa-miR-10a-5p NTA: I-C 3'mod: tg 5'mod: 0
hsa-miR-10a-5p NTA: I-C 3'mod: tg 5'mod: t
hsa-miR-1246 NTA: I-G 3'mod: 0 5'mod: a
hsa-miR-1246 NTA: I-G 3'mod: 0 5'mod: aa
hsa-miR-1246 NTA: I-GA 3'mod: 0 5'mod: 0
hsa-miR-1246 NTA: I-GA 3'mod: 0 5'mod: a
hsa-miR-1246 NTA: I-GAG 3'mod: 0 5'mod: 0
hsa-miR-1246 NTA: I-GAG 3'mod: 0 5'mod: a
hsa-miR-1246 NTA: I-GAG 3'mod: 0 5'mod: aa
hsa-miR-1307-5p NTA: 0 3'mod: gct 5'mod: 0
hsa-miR-143-3p NTA: I-A 3'mod: tc 5'mod: 0
hsa-miR-192-5p NTA: 0 3'mod: c 5'mod: 0
hsa-miR-192-5p NTA: 0 3'mod: c 5'mod: c
hsa-miR-200a-3p NTA: 0 3'mod: t 5'mod: 0
hsa-miR-25-3p NTA: 0 3'mod: C 5'mod: 0
hsa-miR-25-3p NTA: I-C 3'mod: a 5'mod: 0
hsa-miR-27b-3p NTA: 0 3'mod: gc 5'mod: 0
hsa-miR-320a/b/c NTA: 0 3'mod: cga/caa/t 5'mod: 0
hsa-miR-320a/b NTA: 0 3'mod: ga/aa 5'mod: 0
hsa-miR-320a/b/c NTA: I-A 3'mod: cga/caa/t 5'mod: 0
hsa-miR-486-5p NTA: I-T 3'mod: gag 5'mod: 0
hsa-miR-532-5p NTA: 0 3'mod: 0 5'mod: c
hsa-miR-486-5p NTA: I-A 3'mod: gag 5'mod: 0
hsa-miR-320c NTA: 0 3'mod: 0 5'mod: 0
hsa-miR-320c/d NTA: 0 3'mod: gt/a 5'mod: 0
hsa-miR-574-5p NTA: 0 3'mod: gt 5'mod: 0
hsa-miR-95-3p NTA: 0 3'mod: a 5'mod: 0
hsa-miR-375 NTA: 0 3'mod: ga 5'mod: 0
cpm (log2)
Metastasis at Diagnosis
Metastasis
Non-Metastasis

Figure 4.4. MicroRNAs are differentially expressed between rectal cancer patients diagnosed with metastatic disease (stage IV) compared to non-metastatic patients (stage I-III). A) 20 mature miRNAs were significantly associated with metastasis at diagnosis; 18 miRNAs showed higher expression in metastatic patients versus non-metastatc patients and two miRNAs showed lower expression in metastatic patients vs non-metastatic patients B) 47 IsomiRs were differentially expressed between patients diagnosed with metastatic disease vs non-metastatic patients; expression of 40 isomiRs were higher and expression of seven isomiRs were lower in metastatic patients compared to non-metastatic patients.

Next, we investigated the relationship between miRNA expression levels and clinical routine analyses. As shown in (Fig. 4.5-A and B., increased expression of eight mature miRNAs and nine isomiRs were significantly correlated with increasing levels of CEA. Decreasing expression of one isomiR significantly correlated with decreasing CEA levels. Also, high levels of CEA was found significantly assoicated with metastasis at diagnosis in CRC patients (p=0.0007) (Fig. 4.5-C). The two miRNAs miR-451a and miR-877-5p were positively correlated with hemoglobin (Hb) levels. Correlation between miR-451a expression and Hb levels is shown in (Fig. 4.5-D). Correlation of miRNA expression levels with other clinical routine analyses were not found.

Figure 4.5

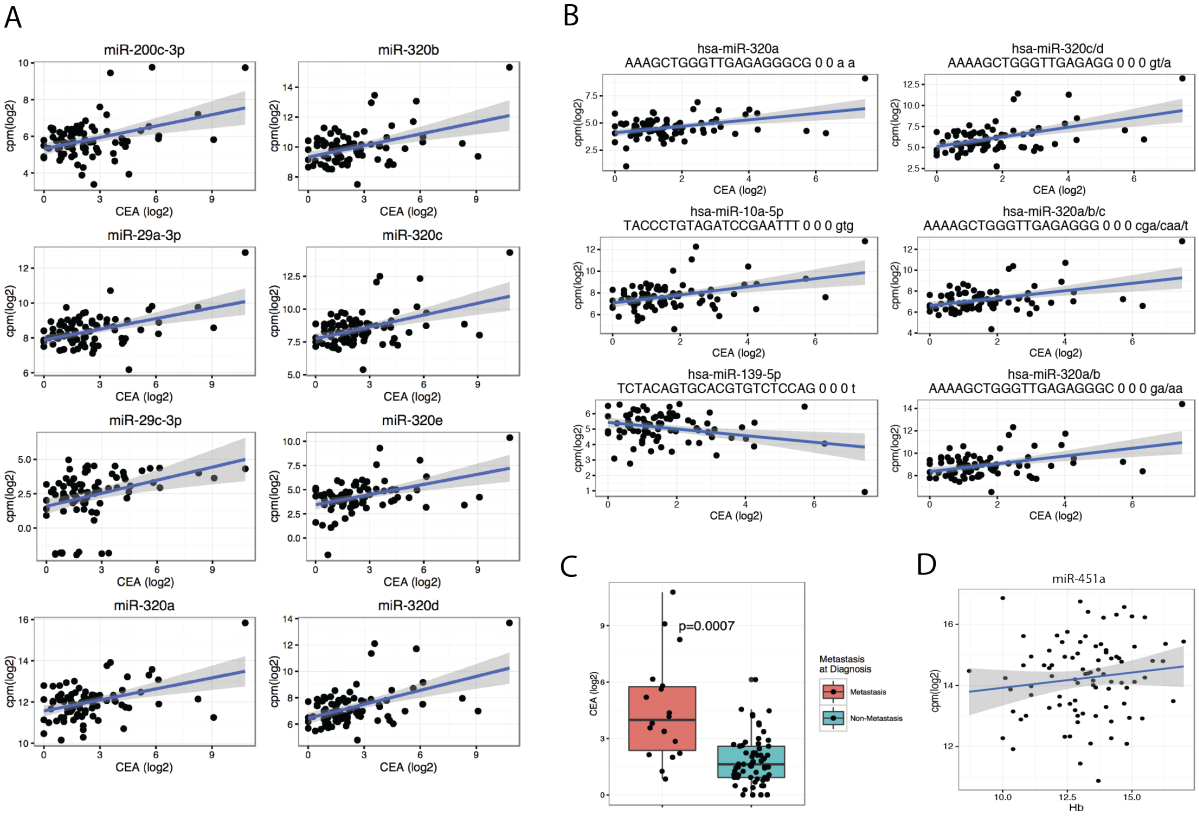


Figure 4.5. Linear regression were used to investigate the relationship of miRNA and isomiR expression with clinical routine analyses and showed that miRNA expression significantly correlated with CEA and Hemoglobin (Hb) levels. A) Scatter plots of the relationship between mature miRNAs and CEA, showing that increasing expression of eight mature miRNAs significantly correlated with increasing CEA levels. B) Nine isomiRs were positively correlated with increasing CEA levels, whereas one isomiR (miR-139-5p) showed an inverse relationship with increasing CEA. C) High CEA levels were significantly associated with metastasis. (stage IV). D) MiR-451a were positively correlated with Hb levels. Plots are shown in log2 values for illustration purpose.

4.5 Functional assay

To validate the findings from Mjelle et al. (unpublished work), the effect on growth of deregulated miRNAs in CRC tissue were investigated using Cell Viability assay in SW480 cells transfected with miRNA mimics, miRNA inhibitors or negative control. Results showed that 14 miRNA inhibitors caused a significant decrease in cell growth compared to negative control ($p<0.05$) (Fig.4.6) No significant effect on cell growth was observed for the miRNA mimics (Fig 4.7).

Figure 4.6

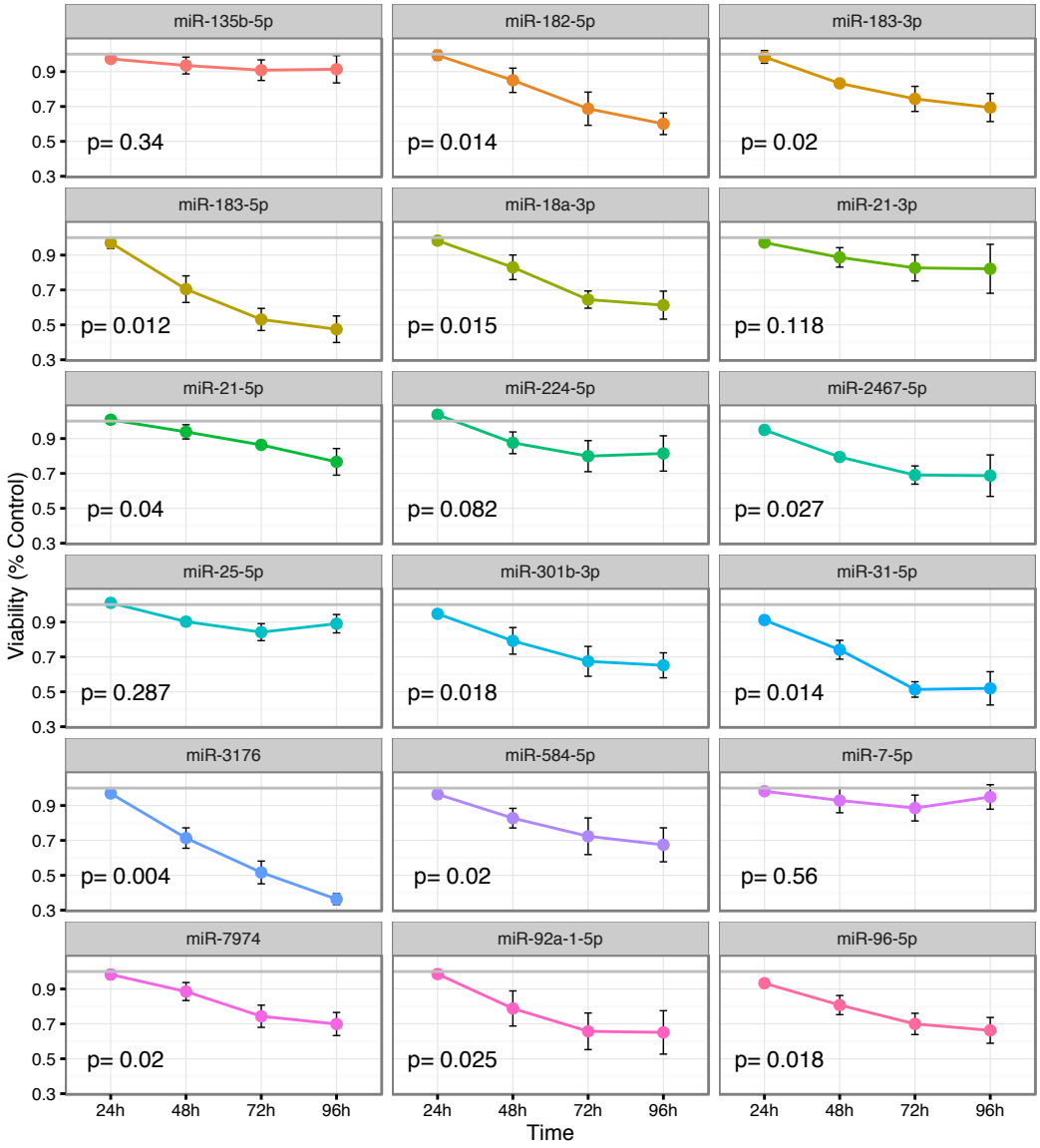


Figure 4.6. CRC cells were transfected with miRNA inhibitors and the number of viable cells were measured after 24, 48, 72 and 96h by Cell Viability assay and fluorescence intensity (FI). 14 miRNA inhibitors had a significant effect on cell growth of SW480 cells compared to negative control (p<0.05) Results are presented as mean with standard error (SE) of three replicates.

Figure 4.7

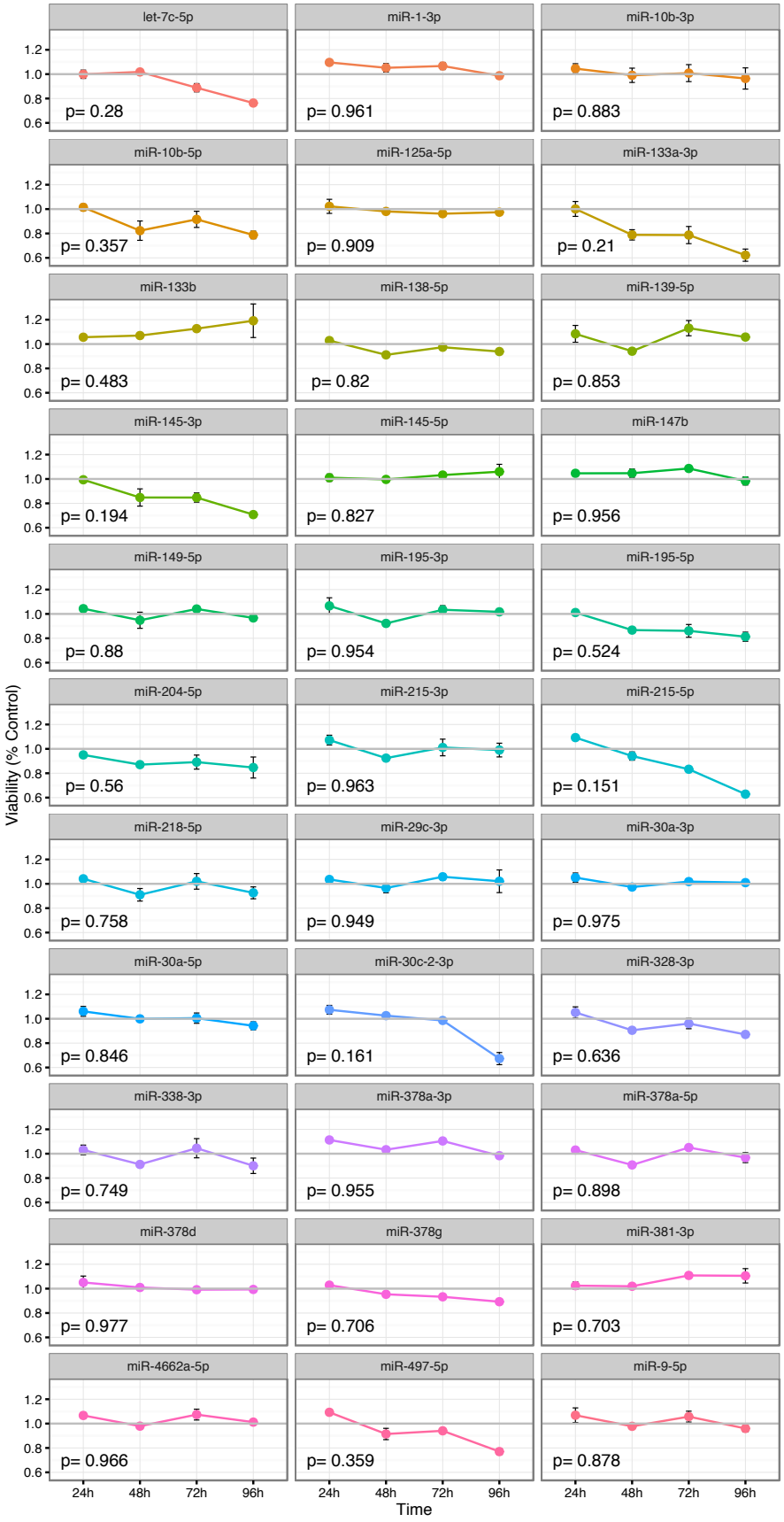

let-7c-5p
p= 0.28
miR-1-3p
p= 0.961
miR-10b-3p
p= 0.883
miR-10b-5p
p= 0.357
miR-125a-5p
p= 0.909
miR-133a-3p
p= 0.21
miR-133b
p= 0.483
miR-138-5p
p= 0.82
miR-139-5p
p= 0.853
miR-145-3p
p= 0.194
miR-145-5p
p= 0.827
miR-147b
p= 0.956
miR-149-5p
p= 0.88
miR-195-3p
p= 0.954
miR-195-5p
p= 0.524
miR-204-5p
p= 0.56
miR-215-3p
p= 0.963
miR-215-5p
p= 0.151
miR-218-5p
p= 0.758
miR-29c-3p
p= 0.949
miR-30a-3p
p= 0.975
miR-30a-5p
p= 0.846
miR-30c-2-3p
p= 0.161
miR-328-3p
p= 0.636
miR-338-3p
p= 0.749
miR-378a-3p
p= 0.955
miR-378a-5p
p= 0.898
miR-378d
p= 0.977
miR-378g
p= 0.706
miR-381-3p
p= 0.703
miR-4662a-5p
p= 0.966
miR-497-5p
p= 0.359
miR-9-5p
p= 0.878
Viability (% Control)
1.2
1.0
0.8
0.6
24h
48h
72h
96h
Time

Figure 4.7. MicroRNA mimics showed no significant growth inhibitory effect on CRC cells. MiRNA mimics had no significant effect on the growth of CRC cells. Results are presented as mean with standard error (SE) of three replicates.

5. Discussion

In the current study, small RNA sequencing was used to identify miRNA isolated from serum of 96 rectal cancer patients to search for potential non-invasive biomarkers in CRC. Herein, we report that a total of 498 mature miRNAs and 3758 isomiRs were detected across all patient samples. Investigation of the relationship between miRNA expression levels and clinicopathological characteristics showed that several mature miRNAs and isomiRs were significantly associated with metastasis (stage IV), CEA levels and overall survival. High levels of the miR-320 family (miR-320a-e), miR-10a-5p, miR-1246 and low levels of let-7b-5p were associated with poor overall survival and metastasis at diagnosis. High levels of miR-320 and miR-10a-5p also showed positive correlation with CEA levels. High levels of miR-200c-3p and miR-29a-3p were associated with metastasis and increasing levels correlated with increasing CEA levels. Not surprising, we found that CEA levels were significantly higher in patients diagnosed with metastatic (stage IV) CRC compared to non-metastatic patients (stage I-III).

Consistent with our results, high levels of miR-200c-3p were previously found in serum of metastatic CRC patients (152). Toiyama and colleagues observed that stage IV CRC patients had significantly higher levels of miR-200c compared to stage I-III patients and high levels were associated with lymph node metastasis, distant disease, liver metastasis, and poor disease-free and overall survival. Our results showed that other members of the miR-200 family (miR-200b-3p/5p and miR-200a-3p) were associated with metastasis. In a recent study on ovarian cancer, serum levels of miR-200a, miR-200b and miR-200c were significantly elevated in patients with advanced disease (174). MiR-29a has previously been detected in serum or plasma of both early (stage I-II) and late stage (III-IV) CRC patients (123,136), but no association between miR-29a and stage I-III were found in our analysis. This may be explained by the fact that we did not include healthy controls and solely focused on miRNA and disease characteristics, whereas Huang et al. compared expression of miR-29a with healthy subjects. Wang et al. found significantly higher levels of miR-29a in serum of metastatic CRC patients and were able to differentiate between metastatic patients and non-metastatic patients with a 75% sensitivity and specificity (175). Circulating levels of the miR-320 family have previously been detected in various cancers, including CRC. (176). Fang et al. analyzed miR-24, miR-320 and miR-423-5p in plasma of CRC patients and showed that this three-miRNA signature could distinguish cases from controls with a sensitivity and

specificity of 92,79% and 70.77%, respectively. They also found that miR-24, miR-320 and miR-423-5p could predict development of metastasis in CRC patients after surgery (177).
In a study by Wang et al., miR-10a-5p were detected at significantly lower levels in plasma of stage II and III CRC patients compared to healthy controls. In contrast to their results, we found that stage IV patients had high levels of miR-10a-5p compared to stage I-III patients. Discrepancies between the two studies might have several explanations, and the use of different patient material (plasma vs serum), different detection methods (qRT-PCR vs high throughput sequencing), and variations between the two study populations could represent some of them. Indeed, different miRNA expressions have been observed between different ethnical groups and tumor locations (160,178). Our study included rectal cancer patients whereas most studies involve both colon and rectal cancer, suggesting that a possible difference in miRNA expression in colon and rectal cancer do not apply to our study. Differing results could also be explained due to the fact that most studies have only investigated canonical miRNA sequences. Sequencing approaches have revealed that pre-miRNAs give rise to a series of sequence variants of different lengths and different 5' and 3' ends during maturation, known as isomiRs (179,180). The canonical sequence might not necessarily represent the most dominant sequence and thus the use of only canonical sequences may underestimate the true miRNA expression in serum. For instance, our study showed that isoforms of miR-10a-5p were significantly associated with survival, metastasis and CEA levels, while the mature sequence were only found to be associated with metastasis.

High levels of miR-1246 were associated with poor overall survival and metastasis in our study. MiR-1246 is generally a much less studied miRNA in cancer, and when conducting a simple search within the PubMed database only 64 articles were found concerning miR-1246 compared to 471 articles on miR-200c. However, recent studies have investigated the expression of circulating miR-1246 in CRC (181,182). Ogata-Kawata and colleagues found significantly elevated levels of miR-1246 in CRC patients compared to healthy subjects and the positive rate of miR-1246 for identification of CRC was 95,5 % compared to 30,7% and 16,0% of CEA and CA 19-9, respectively. Decreased levels of let-7b were previously found in CRC patients with local recurrence compared to non-recurrent patients (183), but no such association was found in our analysis. However, our study was limited to only four patients experiencing local recurrence.

Two miRNAs, miR-877-5p and miR-451a, were correlated with Hb levels. MiR-451a expression have previously been linked to hemoglobin content in blood-derived products (184-186). In a study by Shkurnikov et al., they analyzed the effect of hemolysis on miRNA expression in plasma and found that hemolysis of red blood cells lead to a significant increase in the levels of several miRNAs, including miR-451a (185). Azzouzi et al. used deep sequencing and proteomics to examine the endogenous miRNA expression in human red blood cells and showed that among 197 miRNAs detected, miR-451a were the most abundant and represented >60% of all reads (184). Landoni and colleagues identified 6 miRNAs, including miR-451 to be significantly increased in hemolyzed plasma compared to non-hemolyzed plasma (186). Considering these findings, it is plausible to think that the correlation between miR-451a and hemoglobin levels found in our study is due to lysis of red blood cells in the blood collection tubes, leading to leakage of hemoglobin that would be present in serum after separation from blood cells. It is possible that hemolysis could have altered the expression of other miRNAs detected in our samples and masking the true miRNA expression in serum, possibly leading to an overestimation of the miRNA expression we detected. For instance, Shkurnikov and Landoni et al. also detected increased levels of miR-486-5p in hemolyzed plasma, which were the most abundant miRNA detected in our study. MiR-486-5p is a red blood cell-specific miRNA that is released during hemolysis of red blood cells and can be transferred to serum during separation from blood cells (187). However, none of the other miRNAs identified in their studies were correlated with hemoglobin in our analysis. Additionally, Additionally, is was not made clear from which patients the blood were collected in these studies and the effect could be different in serum from CRC patients. Several other miRNAs have been detected in red blood cells, which could represent a potential confounding when investigating circulating miRNAs (187). It is therefore important that case and control samples are randomly isolated or isolated at the same time, to limit the variation in hemolysis between samples.

The expression levels of three mature miRNAs, namely miR-10b-5p, miR-150-5p and miR-215-5p, differed significantly depending on whether patients had received preoperative treatment or not before serum collection. Levels of miR-10b-5p and miR-215-5p were significantly higher and miR-150-5p levels were significantly lower in the after (preoperative)-treatment group compared to the before-treatment group. In addition, high levels of isomiRs corresponding to miR-125b-5p and miR-30a-5p were observed in the after-treatment group. Previous studies have shown that extracellular miRNA are related to

treatment response in CRC (153,188-191). In a study by Dinh et al, they analyzed miRNA expression in plasma of lung cancer patients undergoing radiotherapy (RT) and found that levels of miR-150-5p and miR-29a-3p were inversely correlated with increasing RT dose. They also found that levels of miR-125b-5p increased with increased RT dose. The potential source of circulating miRNAs were investigated by measuring intra - and extracellular levels (exosomes) of miR-150-5p and miR-29a-3p in irradiated normal and lung cancer cells, and showed that miR-150-5p and miR-29a-3p were decreased in exosomes secreted by irradiated cells while intracellular expression increased upon radiation. (192). Decreased levels of miR-150 have also been found in whole blood of mice exposed to ionizing radiation (193). MiR-125b was recently reported to be able to discriminate between responders and non-responders to preoperative chemoradiotherapy in locally advanced rectal cancer patients (194).

One of the aims of this study was to investigate the effect of deregulated miRNAs in CRC on cell growth. In this regard, CRC cells were transfected with miRNA mimics and inhibitors followed by measurement of viable cells. Our results demonstrated that 14 miRNA inhibitors had a significant negative effect on cell growth. The strongest and most significant effects were observed for miR-3176, miR, miR-183-5p, miR-182-5p, miR-31-5p, miR-18a-3p, miR-96-5p and miR-95-5p. In agreement with our results, members of the miR-183-96-182 cluster are frequently upregulated in cancers, expressed either individually or as a cluster (195), and have been shown to act as oncogenes by targeting tumor suppressors involved in cancer-related pathways, such as cell cycle regulation, cell proliferation, metabolism, apoptosis and angiogenesis (196-202). Upregulation of miR-31 in cancers has been shown to regulate E2F2 transcription factor, which is a major cell cycle regulator (203,204). MiR-95 have been shown to promote cell growth by targeting SNX1 that mediate suppression of EGFR by transporting ligand-activated receptors to lysosomes for degradation, leading to blocked activation of the EGFR/PI3K/Akt pathway (205,206). Although not significant in our experiment, it seemed that inhibition of miR-21-3p caused, at least to some degree, an overall decrease in cell growth. MiR-21-3p is a widely studied oncogenic miRNA that plays an important role in tumorigenesis by its involvement in cancer-related pathways (207,208). We also observed a significant growth reduction for a few non-conserved miRNAs, including miR-2467-5p, miR-3176 and miR-7674, which were recently discovered using deep sequencing. The biological function of these miRNAs remains to be investigated. The miRNAs that were inhibited in the current study are found to be upregulated in many cancers, including colon, prostate and stomach, however, studies have also shown that some of these

miRNAs are also downregulated in cancer. For instance, miR-31 has been found to be downregulated in lung cancer and miR-183 is downregulated in osteosarcoma (209,210), suggesting that miRNAs may have different regulatory roles in different tissues and function as oncomiRs in one cellular context and tumor suppressor miRNAs in others.

In general, we did not observe a significant effect of miRNA mimics on the growth of CRC cells. However, it appeared that some miRNA mimics, e.g. miR-133a-3p, miR-145-3p and miR-215-5p, caused a small effect on growth of SW480 cells. Indeed, several of these miRNAs have previously been reported to be downregulated in cancer and have tumor suppressor activity (211-214). MiRNA inhibitors and miRNA mimics works in two different ways. MiRNA inhibitors are designed to reduce the pool of active miRNAs by forming complementary base pairing with the mature strand of the miRNAs, resulting in less repression of its target genes. MiRNA mimics, on the other hand, are designed to increase repression of target genes by increasing the pool of active miRNAs. A publication by Jin et al. discusses the challenges by using miRNA mimics and points at the potential off-target effects when transfected at high concentrations and lack of target repression when transfected at low concentrations. Moreover, the mimics were often mutated or trimmed/tailed causing off-target effects and unwanted mRNA degradation (215). Since the mimic transfection in the current project were repeated three times with similar results, it is plausible to think that the lack of consistent growth repression could be, at least partly, explained by technical issues with the miRNA mimics.

A potential source of error in our study could have been introduced during RNA isolation. We chose not to use the internal spike-in (*C.elegans* miR-39 miRNA mimic) provided in the QIAGEN kit, because we planned to add calibrator sequences in the library preparation step. In general, variability in recovery of miRNA from serum and plasma can contribute to quantification errors, but comparison of commonly used commercial kits for RNA isolation has shown little variation in RNA recovery (216). Additionally, Bioanalyzer results showed that miRNA were present at acceptable concentrations in our samples.

To our knowledge this is the first study that have investigated the association of clinical and histopathological characteristics with global miRNA and isomiR expression in a large patient cohort. The use of small RNA sequencing allows for the detection of millions of reads at the same time and one of the key advantages is that it allows for the discovery of novel miRNAs

and sequence variants (217). Sequencing is more sensitive and accurate than other widely used methods, such as micro arrays and qualitative real-time PCR (qRT-PCR) (218). However, deep sequencing requires experience in handling big data and statistics, and for optimal comparison of miRNA expression data it is crucial to develop standardized methods in all steps, including sample collection and processing, miRNA assays and processing of data.

In light of previous reports, these results contribute to a better characterization of the expression of circulating miRNAs and the functional role of tissue miRNAs in CRC. We showed that several aberrantly expressed miRNAs in CRC tissue are important players in growth of CRC cells and may possibly be involved in tumor progression *in vivo*. More investigation on their functional roles is needed in order to get a better understanding of the underlying mechanisms of human cancers. Our results show great promise for serum miRNAs as minimally invasive biomarkers in CRC. The miRNAs miR-200c-3p, miR-29a-3p, miR-320a-e, miR-1246, miR-10a-5p and let-7b-5p have potential as prognostic biomarkers in CRC. Generally, metastasis is highly correlated with survival and it is likely that some of the survival-associated miRNAs and isomiRs are significant because of differences in disease stage. However, several miRNAs and isomiRs were found to be associated with survival without being related to metastasis, indicating that other mechanisms may be involved. MiR-10b-5p, miR-215-5p and miR-150-5p, and isomiRs of miR-125b-5p are potential biomarkers for estimating treatment response in CRC patients. Our results demonstrated that isomiR expression largely corresponded with mature miRNAs. However, some isoforms were associated with clinicopathological parameters independently of the canonical sequence, suggesting that isomiRs may provide an additional layer to CRC biomarkers beyond mature miRNAs and that isomiR and miRNA expression should be investigated simultaneously in future experiments. Validation in large-scale studies and specificity and sensitivity of such biomarkers needs to be investigated in order to determine their clinical application.

6. References

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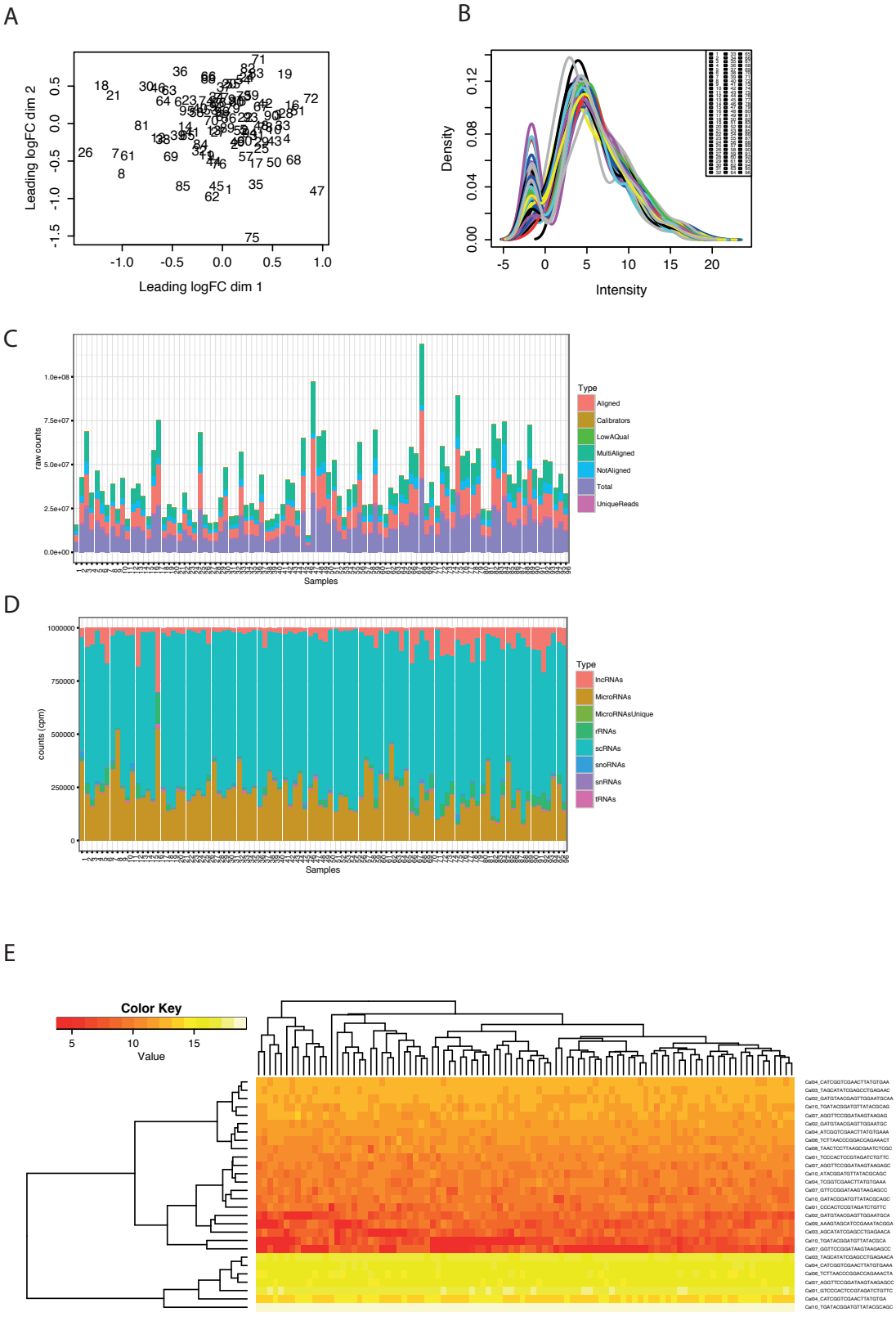
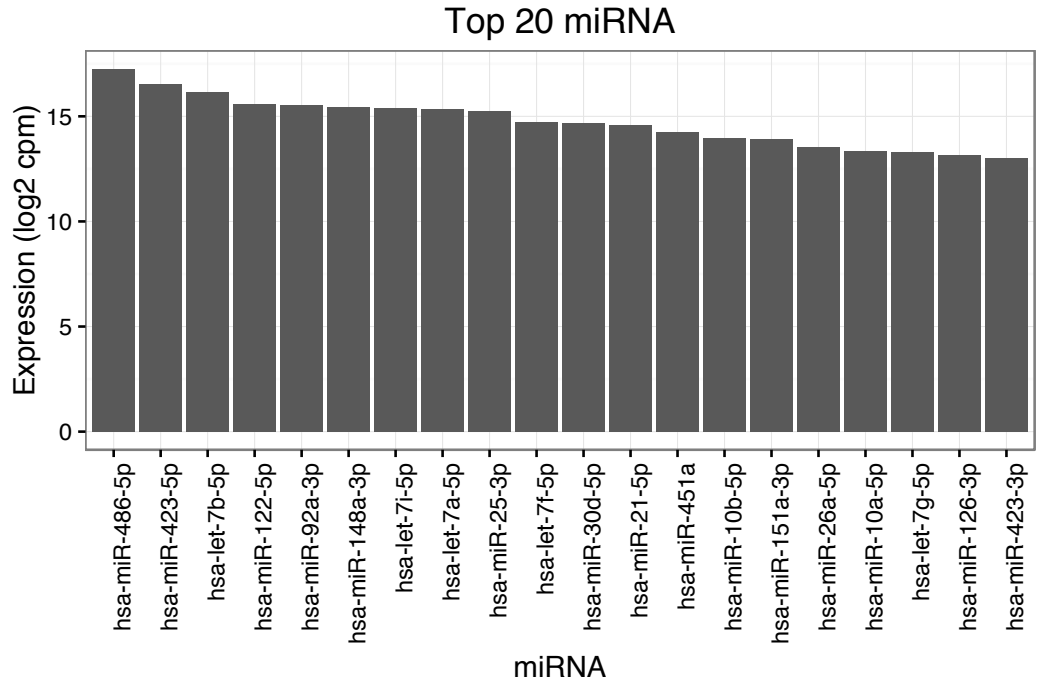
A. Supplementary material

Supplementary figure A.1



Supplementary figure A.1. A) Density plot of spike-in normalized mature miRNAs. The figure shows the density of counts for the different samples. Overlapping densities indicate that the samples are successfully normalized. B) Multidimensional scaling (MDS) plot of normalized miRNA expression in 96 samples. C) Sequencing statistics. Number of reads mapping to the human genome. D) Classes of mapped reads. Reads that successfully mapped to the genome were divided into the main RNA classes. E) Heatmap of normalized spike-ins (cpm, log2). Both the canonical spike-in sequence and processing variants are shown.

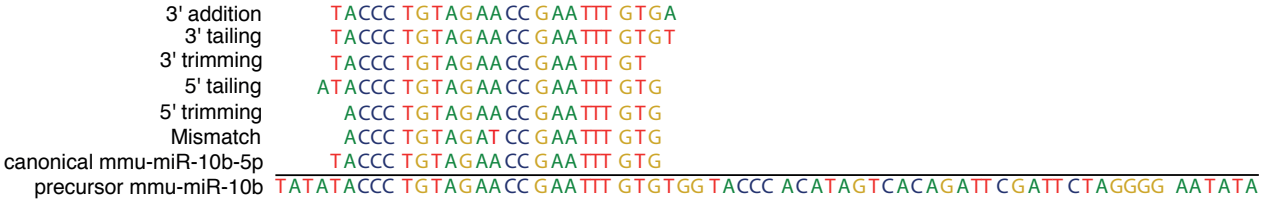
Supplementary figure A.2



Supplementary figure A.2. The top 20 most abundant miRNAs detected across all serum samples from rectal cancer patients.

Supplementary figure A.3

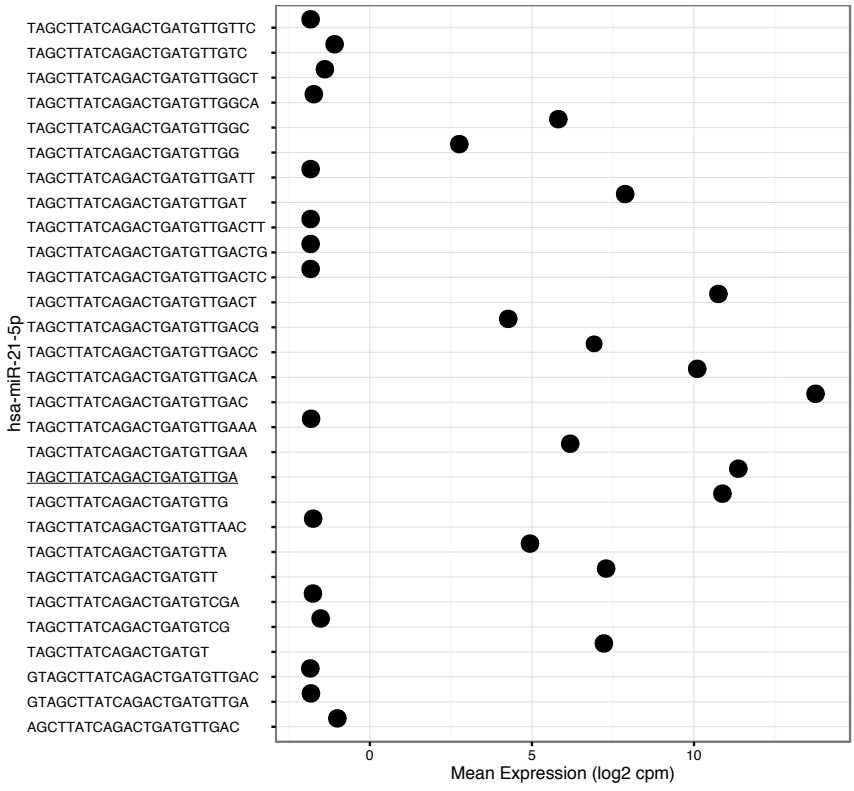
A



B

IsomiR type	Number of IsomiRs
5'tailing	543
5'trimming	272
3'tailing	1897
3'trimming	2663
NTAs	2296
Mismatch	4999

C



Supplementary figure A.3. A) Illustration of the main isomiR types. Sequence variants of miRNA miR-10b-5p is shown as an example. B) Number of isomiRs belonging to the six main isomiR types in the current data. C) Illustration of different isomiRs mapping to the same miRNA strand. Here exemplified by hsa-miR-21-5p.

B. Appendix

B.1 Materials

Appendix table B.1.1 Materials used in this study

Product name	Art.nr/Catalogue nr.	Manufacturer	Lot nr.
Commercial kits			
QIAGEN miRNeasy serum/plasma kit (50) x 2	217184	QIAGEN, Germany	151020493
NEBNext® Multiplex Small RNA Library prep set for Illumina (Set 1)	E7300L	New England BioLabs®, inc., UK	0091504
NEBNext® Multiplex Small RNA Library prep set for Illumina (Set 2)	E7580L	New England BioLabs®, inc., UK	0061504/ 0071510
Technical equipment and instruments			
Mastercycler® EP (Thermal cycler)	- *	Eppendorf, UK	-
NanoDrop ND-1000 Spectrophotometer	-	NanoDrop Technologies, Inc., US	-
2100 BioAnalyzer	G2940CA	Aglient Technologies, US	-
FLUOstar® Omega microplate reader	-	BMG Labtech, Germany	-
Countess™ Automated cell counter	C10227	ThermoFisher Scientific, Invotrogen™, US	-
Cell line and products for cell culture maintenance			
SW480 Colon cancer cell line	-	In-house cell line	-
L-15 Leibovitz Medium, liquid (with L-glutamine)	L1518	Sigma-Aldrich, US	-
Fetal Bovine Serum	F7524	Sigma-Aldrich, US	-
Trypsin (0.2 g EDTA ⍰ 4Na), liquid	T3942	Sigma-Aldrich, US	-
Oxid™ Phosphate Buffered Saline tablets	BR0014G	ThermoFisher Scientific, Thermo Scientific™, US	-
Products used in transfection experiment			
Opti-MEM® Reduced Serum Medium	31985047	ThermoFisher Scientific, Invotrogen™, US	748786
Lipofectamine® 3000 Transfection Reagent	L3000008	ThermoFisher Scientific, Invitrogen™, US	1769228
Trypan Blue stain 0.4%	T10282	ThermoFisher Scientific, Life Technologies, US	1736070
Countess™ cell counting chamber slides	C10228	ThermoFisher Scientific, Invitrogen™, US	2C10413A
PrestoBlue™ Cell Viability reagent	A13261	ThermoFisher Scientific, Life Technologies, US	1710852A
MirVana™ Mimics & Inhibitors[1)]	-	ThermoFisher Scientific, Ambion™, US	-
MirVana™ miRNA mimic negative control #1	4464058	ThermoFisher Scientific, Ambion™, US	-

MirVana™ miRNA inhibitor negative control #1	AM17010	ThermoFisher Scientific, Ambion™, US	-
Other products			
Calibrator sequences[2)]	-	Sigma-Aldrich, US	-
Ethanol Absolute AnalaR NORMAPUR®	20821.310DP	VWR, US	-
Acid Phenol:Chloroform	AM9720	ThermoFisher Scientific, Ambion™, US	1505002

[1)] For more information see Appendix table B.1.3.

[2)] For more information see Appendix table B.1.2.

* For some products there were no article or lot numbers available.

Appendix table B.1.2. Calibrator sequences used as internal standard for small RNA sequencing

Calibrator ID	Sequence
Cal01	GTCCCACTCCGTAGATCTGTTC
Cal02	GATGTAACGAGTTGGAATGCAA
Cal03	TAGCATATCGAGCCTGAGAACA
Cal04	CATCGGTCGAACTTATGTGAAA
Cal05	GAAGCACATTCGCACATCATAT
Cal06	TCTTAACCCGGACCAGAAACTA
Cal07	AGGTTCCGGATAAGTAAGAGCC
Cal08	TAATCTCCTTAAGCGAATCTCGC
Cal09	AAAGTAGCATCCGAAATACGGA
Cal10	TGATACGGATGTTATACGCAGC

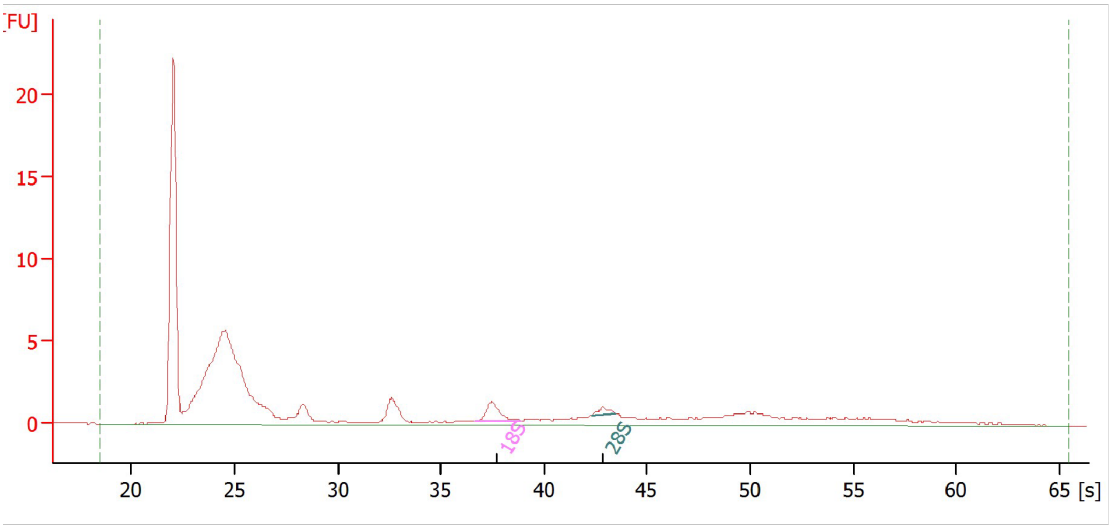
Appendix table B.1.3. MicroRNA mimics and inhibitors used in transfection experiment

Mimics	
MicroRNA (gene symbol)	RefSeq Accession number
hsa-miR-9-5p	MIMAT0000441
hsa-miR-328-3p	MIMAT0000752
hsa-miR-139-5p	MIMAT0000250
hsa-miR-338-3p	MIMAT0000763
hsa-miR-195-3p	MIMAT0004615
hsa-miR-4662a-5p	MIMAT0019731
hsa-miR-133a-3p	MIMAT0000427
hsa-miR-378g	MIMAT0018937
hsa-miR-378a-5p	MIMAT0000731
hsa-miR-29c-3p	MIMAT0000681
hsa-miR-30a-3p	MIMAT0000088
hsa-miR-133b	MIMAT0000770
hsa-miR-195-5p	MIMAT0000461

hsa-miR-145-5p	MIMAT0000437
hsa-miR-30a-5p	MIMAT0000087
hsa-miR-138-5p	MIMAT0000430
hsa-miR-378a-3p	MIMAT0000732
hsa-miR-30c-2-3p	MIMAT0004550
hsa-miR-1-3p	MIMAT0000416
hsa-miR-147b	MIMAT0004928
hsa-miR-497-5p	MIMAT0002820
hsa-miR-215-5p	MIMAT0000272
hsa-miR-10b-5p	MIMAT0000254
hsa-miR-215-3p	MIMAT0026476
hsa-miR-145-3p	MIMAT0004601
hsa-miR-218-5p	MIMAT0000275
hsa-miR-149-5p	MIMAT0000450
hsa-miR-381-3p	MIMAT0000736
hsa-miR-204-5p	MIMAT0000265
hsa-miR-10b-3p	MIMAT0004556
hsa-miR-378d	MIMAT0018926
hsa-miR-125a-5p	MIMAT0000443
hsa-miR-let-7c-5p	MIMAT0000064
Inhibitors	
hsa-miR-21-3p	MIMAT0004494
hsa-miR-135b-5p	MIMAT0000758
hsa-miR-584-5p	MIMAT0003249
hsa-miR-7974	MIMAT0031177
hsa-miR-183-3p	MIMAT0004560
hsa-miR-182-5p	MIMAT0000259
hsa-miR-183-5p	MIMAT0000261
hsa-miR-21-5p	MIMAT0000076
hsa-miR-31-5p	MIMAT0000089
hsa-miR-301b-3p	MIMAT0004958
hsa-miR-92a-1-5p	MIMAT0004507
hsa-miR-7-5p	MIMAT0000252
hsa-miR-25-5p	MIMAT0004498
hsa-miR-3176	MIMAT0015053
hsa-miR-224-5p	MIMAT0000281
hsa-miR-18a-3p	MIMAT0002891
hsa-miR-2467-5p	MIMAT0019952
hsa-miR-96-5p	MIMAT0000095

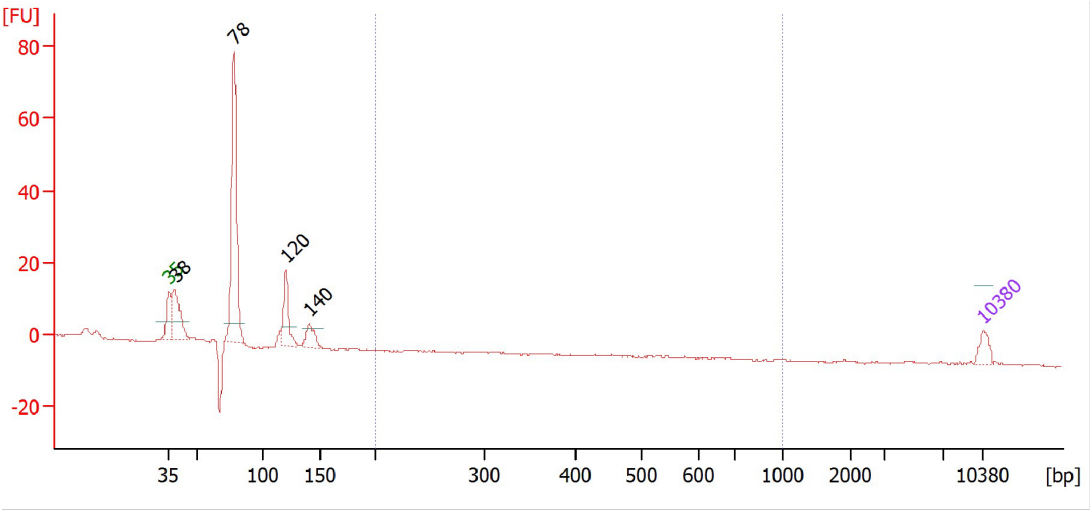
B.2 Bioanalyzer results for quantification and quality assessment of RNA isolation and small RNA cDNA library preparation

Appendix figure B.2.1



Appendix figure B.2.1. Bioanalyzer result from isolated RNA. Result from one of the samples analyzed on Bioanalyzer. Data are translated into an electropherogram where migration time versus intensity is plotted with the help of an external standard. Migration time is correlated with fragment size. A peak around 25s is indicative of small RNA present in the sample.

Appendix figure B.2.2



Appendix figure B.2.2. Bioanalyzer result from cDNA library. Result from one of the samples analyzed on Bioanalyzer. Data are translated into an electropherogram where migration time versus size (bp) is plotted with the help of an internal standard. A peak around 142 bp is indicative of cDNA in the sample.

B.3 TNM staging system and stage groups

Appendix table B.3.1. AJCC (American Joint Committee on Cancer) TNM staging system 5th edition*

T (tumor invasion)	Description
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*; intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum
N (spread to regional lymph nodes)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes
M (occurrence of distant metastasis)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Appendix table B.3.2. AJCC TNM Stage grouping 5th edition**

Stage group	Tumor invasion	Lymph node metastasis	Distant metastasis
Stage 0	Tis	N0	M0
Stage I	T1-T2	N0	M0
Stage II	T3-T4	N0	M0
Stage III	Any T	N1-N2	M0
Stage IV	Any T	Any N	M1

*/** Tables are modified from AJCC Cancer Stagning Manual 5th edition (219).