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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

MicroRNA in rectal cancer

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Abstract

In rectal cancer, one of the most common cancers worldwide, the proper staging of the disease determines the subsequent therapy. For those with locally advanced rectal cancer, a neoadjuvant chemoradiotherapy (CRT) is recommended before any surgery. However, response to CRT ranges from complete response (responders) to complete resistance (non-responders). To date we are not able to separate in advance the first group from the second, due to the absence of a valid biomarker. Therefore all patients receive the same therapy regardless of whether they reap benefits. On the other hand almost all patients receive a surgical resection after the CRT, although a watch-and-wait procedure or an endoscopic resection might be sufficient for those who responded well to the CRT. Being highly conserved regulators of gene expression, microRNAs (miRNAs) seem to be promising candidates for biomarkers. Many studies have been analyzing the miRNAs expressed in rectal cancer tissue to determine a specific miRNA profile for the ailment. Unfortunately, there is only a small overlap of identified miRNAs between different studies, posing the question as to whether different methods or differences in tissue storage may contribute to that fact or if the results simply are not reproducible, due to unknown factors with undetected influences on miRNA expression. Other studies sought to find miRNAs which correlate to clinical parameters (tumor grade, nodal stage, metastasis, survival) and therapy response. Although several miRNAs seem to have an impact on the response to CRT or might predict nodal stage, there is still only little overlap between different studies. We here aimed to summarize the current literature on rectal cancer and miRNA expression with respect to the different relevant clinical parameters.

Key words: Polymorphism; MicroRNA; Rectal cancer; Response; Chemoradiotherapy; Expression

Core tip: In rectal cancer, a proper staging of the disease determines the subsequent therapy. Also, prediction of prognosis or therapy response could serve to individualize therapy. MicroRNAs (miRNAs) are highly conserved

regulators of gene expression, and seem to be promising candidates for biomarkers. Several miRNAs are part of a specific expression profile in rectal cancer tissue, while others have been correlated to clinical parameters and therapy response. However the comparison of different studies shows only little overlap and even partly oppositional results. Differences between analytical methods and tissue storage types can contribute to that. Further functional analyses are needed to fully understand the impact of miRNAs in rectal cancer.

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INTRODUCTION

Colon and rectal cancer

Taken together, colon and rectal cancer is the third most common cancer worldwide, accounting for 1.36 million newly diagnosed colorectal cancers in 2012[1] with rectal cancer accounting for 30%. The main purposes to differentiate between colon and rectal cancer are anatomical[2] and molecular differences[3]. Several studies have also shown that disease-correlated genetic and lifestyle factors differ between colon and rectal cancer[3-8]. Differences in survival, fewer inherited syndromes, and younger age at diagnosis in rectal cancer patients further strengthen the rationality of separating the two diseases[8].

Specifically due to the anatomical differences in comparison to colon cancer, local recurrence is a considerable concern in the treatment of rectal cancer. This led to the introduction of radiation in the treatment of rectal cancer patients and represents fundamental therapeutic differences to colon cancer. While treatment of upper rectal cancer provides primary surgical resection and can therefore be compared to colon cancer, the standard treatment of locally advanced cancer in the lower and middle rectum includes preoperative chemoradiotherapy (CRT), followed by total mesorectal excision (TME)[9]. The introduction of preoperative chemoradiotherapy requires additional challenges in diagnostics and therapy planning which differ from colon cancer, requiring a precise pretherapeutic staging.

The rectal cancer staging can be made according to the TNM staging system from the World Health Organization: The Union for International Cancer Control (UICC). Depending on tumor status, nodal status, and metastases, rectal cancer is subdivided in UICC Ⅰ-Ⅳ. While tumor status is determined by magnetic resonance imaging and trans-rectal endoscopic ultrasound, metastasis status is assessed by computed tomography of thorax and abdomen and ultrasound of the latter. Defining nodal status remains the most challenging and is evaluated today using all aforementioned imaging techniques. Correct staging of patients with rectal cancer is actually required at two time points: First, before starting any treatment, and second, after neoadjuvant chemoradiotherapy, because response to CRT is heterogeneous; it ranges from resistance to complete pathological response. Response to CRT, measured as tumor regression grade (TRG), correlates significantly with disease-free- and overall-survival. The first staging is crucial for deciding if a preoperative CRT is needed, hence only locally advanced stages receive CRT. The second staging acquires more and more importance with regard to the possibility of organ-preserving strategies, which have been recently suggested as an alternative to TME for patients that responded very well to CRT. Basis for this upcoming approach can be found in the side effects of rectal cancer surgery. However, if lymph node metastases are undetected these have to be considered as origin of local relapse. In this respect molecular markers may play an increasing role as potential predictive marker.

miRNAs

MicroRNAs (miRNAs) are short non-coding RNAs, 20-22 nucleotides in length discovered in 2001[10,11]. They are highly conserved between vertebrates, invertebrates and plants[12]. Through base-pairing with their target mRNA, miRNAs induce post-transcriptional gene silencing by mRNA degradation or translational blocking[13,14]. Consequently, they present master regulators of gene expression and therefore influence many physiological and patho-physiological processes[15].

Some conservatively estimated 60% of all human mRNAs are regulated by miRNAs, which represent virtually all cellular and molecular functions. Thus, it is not surprising that miRNAs are involved in diverse processes including embryonic development, cell differentiation, cellular proliferation, metabolism, adaptation to environmental stress, and apoptosis[13]. Thus, miRNAs play important roles in many human diseases, and even in the human aging process[16]. By now the impact of specific miRNAs is reported not only for almost every cancer type but also for other diseases like diabetes, cardiovascular diseases, neurological diseases and even psychological diseases like schizophrenic disorder. Therefore miRNAs are of great interest as possible biomarkers in various diseases due to their abundance and cell-type specificity.

Many human miRNA loci are located within intronic (miRtrons) regions[17,18]. While it is a general belief that intronic miRNAs are released from excised introns after the splicing, an interesting study of Kim *et al*[19] indicates that intronic miRNAs can be processed from unspliced intronic regions, ensuring both miRNA biogenesis and protein synthesis from a single primary transcript, supporting the assumption that the intronic miRNAs and their hosting genes are co-regulated[20]. miRNAs are transcribed by RNA Polymerase Ⅱ (pol Ⅱ). The primary transcripts (pri-miRNA) are 5′-capped and polyadenylated. They have at least one stem-loop structure that encodes an individual miRNA sequence within the stem. Drosha, a nuclear RNase Ⅲ type enzyme, and DGCR8, a double-

stranded RNA-binding protein, work as a complex known as microprocessor, which cleaves the primary structure of the pri-miRNA in a process called "cropping"[10]. The products of this reaction are the pre-miRNs, which are exported to the cytoplasm by exportin-5[21,22]. In the cytoplasm the pre-miRNAs are further processed by the cytoplasmic RNase Ⅲ called Dicer. Only one strand of the produced duplex of RNA is incorporated into the effector complex RNA-induced silencing complex (RISC), acting as the guide strand, while the passenger strand is rapidly degraded. However, either arm of the pre-miRNA can be selected to become the guide strand. The strand-selection differentially or coherently processes mature miRNAs, giving rise of gene regulatory RNAs with distinct target-spectra. This process of flexible arm selection has been reported for many small RNAs, including canonical and intronic miRNAs[23,24]. Eventually, RISC migrates to P-bodies to scan and bind to the 3′ untranslated region of the target mRNA.

The miRNA-mRNA binding is specific due to the sequence complementarity of the "seed" region of the miRNA. The canonical seed is a tract of 7-8 nucleotides usually located at the 5′ end of the miRNA molecule, which is fully base pairing one or multiple sites within the sequence of a target mRNA[25] capable of follow structures of miRNA-5"-seeds or alternative seed architectures.

Hence, the core of the target-region with high complementarity is short, which results in multitude of transcripts with possible binding sites for a given miRNA. Therefore a single miRNA has the potential to regulate hundreds of different mRNA targets[26], while on the other hand a single mRNA is regulated by diverse miRNAs simultaneously.

Genome-wide miRNA-expression-profiling studies have demonstrated a specific profile of upregulated and downregulated miRNAs in almost all cancer types[27,28]. In particular, due to their lack of complex post-transcriptional modifications in contrast to mRNAs and other RNA classes (rRNA, tRNA), the potential of miRNAs as biomarkers for cancer diagnosis, prognosis, and response to treatment is expected high. Not only miRNAs can be found in serum or plasma of patients and healthy individuals, but also in other body fluids such as tears, breast milk, bronchial lavage, colostrum and seminal, amniotic cerebro-spinal, pleural and peritoneal fluids[29]. The diagnostic potential of miRNAs relies in part on their stability to storage handling: miRNAs remain stable even in conditions most RNAs would normally degrade (extreme pH-levels, boiling, *etc.*)[30].

Cell-free and circulating miRNAs can be vesicle associated (exosomes and microvesicles), or stable AGO-miRNA complexes and became well accepted biomarkers for non-invasive biomarkers for numerous cancer types[31-36].

LITERATURE SEARCH

A systematic literature search was conducted using PubMed for "rectal cancer", "miRNA" and "miRNA". A total of 27 studies containing miRNA research from rectal cancer tissue, normal mucosa tissue, and body fluids were included for review. Five studies were involved in differential expression of miRNAs in rectal cancer, six studies explored specific miRNAs which showed a correlation to clinical parameters, and nine studies analyzed miRNAs concerning alteration during chemoradiotherapy and response prediction. Five studies conducted further *in vitro* analyses for rectal cancer specific miRNAs. Three studies were found to be dealing with polymorphism in miRNAs in rectal cancer patients.

DIFFERENTIAL EXPRESSION OF MIRNA IN RECTAL CANCER

It is widely accepted that tumors share specific oncogenic pathways. *Vice versa* the tissue of origin has also an impact on the molecular features of each tumor. These are of great interest as they may explain cellular processes such as carcinogenesis, progression, or therapy resistance. Accordingly, rectal cancer specimens and normal mucosa tissue were analyzed. In a first analysis Slattery *et al*[8] compared colorectal cancer tissue [formalin-fixed paraffin embedded (FFPE)] to normal tissue samples and also compared normal rectal tissue to normal colon tissue using microarray analysis. All samples were further subdivided according to their CpG island methylator phenotype status or the mutational status of *KRAS* or p53, revealing 129, 143, and 136 unique miRNAs respectively. The availability of miRNA expression data of normal colon and rectal tissue samples enabled a comprehensive comparison, which identified 73 differentially expressed genes (based on a two-fold fold change) and thus highlighted also important molecular differences between colon and rectal cancer. A comparable study by Li *et al*[37] involving miRCURY Array LNA miRNA chips and technical validation by RT-PCR analyzed expression profiles from six rectal cancer tissues and paired adjacent non-tumor tissue, which identified 67 upregulated and 39 downregulated miRNAs associated with rectal cancer. The number of rectal cancer tissues used (n = 6) is extremely low and, by using an array platform with several hundreds of miRNAs, it is required to correct for multiple testing. This did not occur; therefore the findings are potentially inapplicable.

In a larger study, our own group[38] used LNA-enhanced miRCURY microarrays to map the expression of 2090 miRNAs. Tumor biopsies and matched mucosa samples of 57 patients with locally advanced rectal cancer were profiled. Forty-nine miRNAs differed with high significance between normal and rectal cancer tissue, 20 of these 49 miRNAs were upregulated while 29 were downregulated in rectal cancer *vs* mucosa. Upon employing a combination of fold-change and *P*-value for selection, the expression of 10 miRNAs was validated using 48 samples (24 matched tumor-mucosa samples) by semi-qRT-PCR; in 8 of the 10 miRNA expression levels correlated very well with miRCURY data as they showed the same alteration in both methods and both sets of tissue.

Studies by Wang *et al*[39] could confirm that the expression level of two miRNAs (miR-34a, miR-200c) that were previously found to be differentially regulated in various types of cancer, also were significantly upregulated in rectal cancer by analyzing 72 rectal cancer samples *via* qPCR.

Comparison of different studies to identify overlapping miRNA expression differences, *e.g.*, between rectal cancer and normal tissue is subject of certain restriction: Starting from tissue retrieval (*e.g.*, taking the biopsy during rectoscopy *vs* tissue excision from the resected surgical specimen that obviously already has a certain ischemia time) over tissue storage (*e.g.*, liquid nitrogen, RNA later, formalin fixation) and tissue work up to the final application of the various techniques that are available for miRNA measurement (*e.g.*, miRNA arrays from different companies, qPCR, sequencing). In this specific application, the reference tissue is of importance. Biases arise depending on whether paired normal mucosa or mucosa from different patients were used as a reference. On the other hand, miRNAs that finally overlap between different studies attract attention as they may be represent basic differences between the compared tissues. In this respect we aimed to identify the overlap between published data sets that were previously introduced, which currently involve only two relevant datasets comparing rectal cancer and normal tissue[37,38]. Of these, 11 miRNAs were overlapping. Seven miRNAs were significantly upregulated (miRNAs 17, -18a, -21, -31, -135b, -223 and -492) while four were significantly downregulated (miRNAs-29c, -145, 147b and -375). In both studies also the expression of let-7f, miR-148 and -190 were significantly altered in rectal cancer, however they showed an oppositional regulation of these miRNAs comparing with the first two studies questioning their relevance for assessing differentially expression. For miR-145 even a third study performed by Wang *et al*[40] confirmed a significant in rectal cancer. Figure 1 shows an overview about the differential expression of miRNAs found according to the mentioned studies.

A closer look to the differentially expressed miRNAs reveals a broad range of different function. As a member of the miR-17/92 cluster miR-17 and 18a are both known to be involved in a large number of processes including normal development, tumorigenesis, immune-, cardiovascular-, and neurodegenerative diseases as well as aging[41]. Renal fibrosis[42], myelodysplastic syndroms[43], inflammatory processes[44], and especially cancer are only a few processes that are regulated by miR-21[2,45,46]. For miR-31 a decent number of cancer related studies have been published[47] indicating a more aggressive disease of colorectal cancer[48] if highly expressed. However, a relation to metastastic disease[47,49] and an inverse meaning of increased expression status has been shown in other cancer entities such as breast cancer[48]. The presence of higher expression in different cancer types was reported for miR-135b as well. Nonetheless, it was predominantly analyzed in colorectal cancer and its overexpression by APC loss, PTEN/PI3K pathway deregulation, and SRC overexpression was demonstrated to promote tumor transformation and progression[50]. An oncogenic functionally relevant expression has also been found for miR-223 showing a wide range of different tumor entities[51,52]. In contrast to previous miRNAs, data on the function of miR-492 and its oncogenic relevance are rare. Downregulation of miR-29c - a member of the miR-29 family - is known in several cancer types and its role as a tumor suppressor has been established[53]. Furthermore, its relevance as antifibrotic miRNA is under debate[54]. Initial functional relevance of miR-375 was found as a pancreatic islet-specific miRNA. Recently, miR-375 has been found significantly downregulated in multiple types of cancer, targeting several important oncogenes like AEG-1, YAP1, IGF1R and PDK1[55]. miR-145 is presumed to be a tumor suppressor with apoptosis inhibitor 5, ERK5, K-RAS, and insulin receptor substrate 1 as predicted targets, which are cell cycle and survival regulators[56]. Data on miR-147 is rare; one study postulates that miR-147 is induced upon Toll-like receptor stimulation and regulates murine macrophage inflammatory responses[57]. Taken together, the identified miRNAs from both studies revealed functionally characterized regulators that have, in the vast majority, no organ specificity.

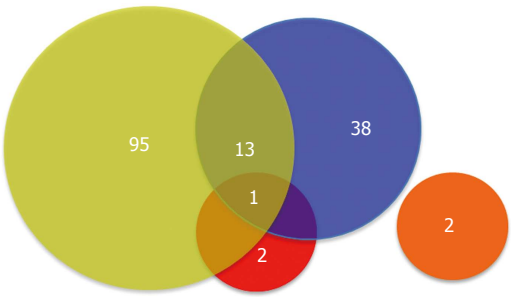
CORRELATION OF MIRNA EXPRESSION TO CLINICAL PARAMETERS

Currently, the most reliable tumor marker to assess clinical outcome is the staging system by TNM classification. As this classification is now more than 100 years old, molecular features for different tumor entities are increasing in number markers for a more precise prognosis are expected. In this respect the aforementioned study of Gaedcke *et al*[38] identified miR-135b. Its expression correlated significantly with disease-free and cancer-specific survival in an independent cohort of 116 patients. miR-135b was also found by other groups to be of importance. Xu *et al*[58] used frozen tissues, performed qPCR analysis, and found miR-135b to have the highest fold-change (17.7-fold) among the upregulated miRNAs in Duke stage Ⅳ cases (that are known to be of poor prognosis). They also identified miR-145 to be highly downregulated with a negative fold change between 18 and 23 in stages Ⅱ, Ⅲ and Ⅳ CRC respectively. Furthermore, they identified significantly decreased expression miR-374a for the identification of patients without metastasis, its effectiveness was confirmed with a sensitivity of 93.33% but a low specificity of only 66.67%. miR-4634 was related to lymph node metastasis in stage Ⅲ with a sensitivity of 75% and specificity of 83.33%. In this analysis, however, the limitation of a mixed study population of colon and rectal cancer must be acknowledged.

Slattery *et al*[59] analyzed data from 1141 CRC cases *via* microarray to identify the impact of 121 miRNAs on disease stage and survival. Five miRNAs were associated with advanced disease stage: hsa-miR-145-5p and hsa-

Li *et al*[37] 2012			
miR-17/18a/18b	Upregulated	miR-1274a	Upregulated
miR-21	Upregulated	miR-10a	Upregulated
miR-31	Upregulated	miR-33a	Upregulated
miR-135b	Upregulated	miR-93	Upregulated
miR-223	Upregulated	miR-98	Upregulated
miR-492	Upregulated	miR-452	Upregulated
miR-19a/20a/a*	Upregulated	miR-886-3p	Upregulated
miR-886-5p	Upregulated	miR-27a	Upregulated
miR-374a	Upregulated	miR-1308	Upregulated
miR-1259	Upregulated	miR-9*	Upregulated
miR-32	Upregulated	miR-106a	Upregulated
miR-196b/196b*	Upregulated	miR-421	Upregulated
miR-96	Upregulated	miR-424	Upregulated
miR-190	Upregulated	miR-1201	Upregulated
miR-182	Upregulated	miR-7	Upregulated
miR-362-3p	Upregulated	miR-183	Upregulated
miR-335	Upregulated	miR-15a	Upregulated
miR-552	Upregulated	miR-106a	Upregulated
Let-7f	Upregulated	miR-200a	Upregulated
miR-301a	Upregulated	miR-339-5p	Upregulated
miR-491-3p	Upregulated	miR-24-2*	Upregulated
miR-140-5p	Upregulated	miR-148a	Upregulated
miR-181d	Upregulated	miR-15b	Upregulated
miR-16	Upregulated	miR-92a	Upregulated
miR-203	Upregulated	miR-25	Upregulated
miR-126	Upregulated	miR-10a	Upregulated
miR-142-3p	Upregulated	miR-30c-1*	Upregulated
miR-374b	Upregulated	miRPlusE1016	Upregulated
miR-29*/a*/b	Upregulated	miRPlusA1087	Upregulated
miR-141*	Upregulated		

Li *et al*[37] 2012	
miR-29c	Downregulated
miR-145	Downregulated
miR-147b	Downregulated
miR-375	Downregulated
miR-1973	Downregulated
miR-378/378c	Downregulated
miR-7-2*	Downregulated
miR-513a-5p	Downregulated
miR-640	Downregulated
miR-381	Downregulated
miR-133b	Downregulated
miR-186*	Downregulated
miR-494	Downregulated
miR-125b	Downregulated
Let-7b/d	Downregulated
miR-139-5p	Downregulated
miR-1184	Downregulated
miR-155	Downregulated
miR-320a-d	Downregulated
miR-150	Downregulated
miR-518e/519a	Downregulated
miR-519b/519c	Downregulated
miR-522/523	Downregulated
miR-125a-5p	Downregulated
miRPlus-E1067	Downregulated
miRPlus-F1027	Downregulated
miRPlus-E1033	Downregulated
miRPlus-E1238	Downregulated
miRPlus-E1173	Downregulated
miRPlus-1285	Downregulated
miRPlus-F1026	Downregulated
miRPlus-E1101	Downregulated
miRPlus-F1155	Downregulated
miRPlus-F1215	Downregulated
miRPlus-E1172	Downregulated
miRPlus-C1110	Downregulated
miRPlus-F1215	Downregulated
miRPlus-E1192	Downregulated



Wang *et al*[39] 2012	
miR-34a	Upregulated
miR-200c	Upregulated

Wang *et al*[40] 2009	
miR-145	Downregulated
miR-143	Downregulated
miR-31	Upregulated

Gaedcke *et al*[38] 2012			
miR-17	Upregulated	miR-29c	Downregulated
miR-18a	Upregulated	miR-143/-145	Downregulated
miR-21	Upregulated	miR-147b	Downregulated
miR-31	Upregulated	miR-375	Downregulated
miR-135b	Upregulated	miR-1	Downregulated
miR-223	Upregulated	miR-30b/c/e	Downregulated
miR-492	Upregulated	miR-26a/b	Downregulated
miR-135b	Upregulated	miR-190	Downregulated
miR-144	Upregulated	miR-192/192*	Downregulated
miR-483-5p	Upregulated	miR-195	Downregulated
miR-542-5p	Upregulated	miR-338-3p	Downregulated
miR-584	Upregulated	miR-10	Downregulated
miR-652	Upregulated	miR-100	Downregulated
miR-675	Upregulated	miR-101	Downregulated
miR-1077	Upregulated	miR-let7a/f	Downregulated
miR-2110	Upregulated	miR-378	Downregulated
miRPlus-E1234	Upregulated	miR-215/194	Downregulated
miRPlus-E1245	Upregulated	miR-429/200b	Downregulated
miRPlus-E1079	Upregulated	miR-148a	Downregulated
miRPlus-A1027	Upregulated	miR-342-3p	Downregulated
		miRPlus-E1141	Downregulated

Figure 1 Differential expression of microRNAs in rectal cancer. The differentially expressed microRNAs (miRNAs) in rectal cancer compared to normal rectal tissue are listed, sorted by studies, respectively. The correlating circles show the number of differentially expressed miRNAs in the mentioned studies and point out the number of miRNAs overlapping between those studies.

miR-31-5p were increased and hsa-miR-200b-3p, hsa-miR-215 and hsa-miR-451a were decreased in advanced stages of CRC. In rectal cancer, 13 miRNAs were significantly associated with mortality after a diagnosis with rectal cancer (Table 1). In addition, they showed that miR-21 expression had an inverse association with mortality in rectal cancer (but not colon cancer patients). However, Nielsen *et al*[60] used *in situ*-hybridization and real-time qPCR on FFPE tissue, and identified miR-21 to predict a short disease-free survival in colon cancer, but not in rectal cancer. Interestingly, the *in-situ*-hybridization showed that the miR-21 expression was detected predominantly in the stromal compartment of the tumors. Yang *et al*[61] showed in an microarray analysis of samples from 40 patients a significant overexpression of miR-21, miR-155, miR-29a and miR-92a in rectal cancer samples and found only miR-155 had the capacity to discriminate nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages.

Stratmann *et al*[62] did not investigate miRNAs directly but the expression level of Dicer - one of the key enzymes in the miRNA generating process - and revealed that the Dicer expression in rectal cancer is higher than in normal mucosa (and higher than in colon cancer), while Dicer expression in liver metastases was decreased in comparison to either the primary tumor or mucosa. Furthermore, patients with a high expression of Dicer mRNA in the normal mucosa had a worse prognosis (poor survival) than those with a lower expression level.

ALTERATION DUE TO THERAPY AND PREDICTING THERAPY RESPONSE

While imaging techniques (computer tomography, magnetic resonance imaging and ultrasound) manage to diagnose tumor stage, nodal stage or distant metastasis initially in an appropriate manner, their ability to identify the response after chemoradiotherapy is poor, particularly the differentiation between vital tumor cells and scar tissue is challenging for imaging techniques. Response to neoadjuvant chemoradiotherapy measured as TRG is therefore usually determined by pathologists after investigating the operative specimen. An adequate

Table 1 Association of microRNA expression and clinical parameters

microRNA	Clinical parameter	Change of expression	Ref.
miR-17-5p miR-20a/20b-5p miR-21-3p/5p miR-25-3p miR-29a/29c-3p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-31-5p	Association with advanced tumor stage	Increased expression level in advanced tumor stage	Slattery *et al*[59]
miR-135b	Correlation with disease-free and cancer-specific survival	Patients with a high expression level of miR-135b had a better disease-free and cancer-specific survival	Gaedcke *et al*[38]
miR-135b	Correlation with Duke stage Ⅳ	Upregulated with the highest fold-change (17.7-fold) among 9 upregulated miRNAs	Xu *et al*[58]
miR-141-3p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-145	Correlation with Duke stage Ⅱ, Ⅲ, Ⅳ	Downregulated with a -18.15, -18.9, -23.8-fold change in stage Ⅱ, Ⅲ and Ⅳ CRC respectively	Xu *et al*[58]
miR-145-5p	Correlation with advanced tumor stage	Increased expression level in advanced tumor stage	Slattery *et al*[59]
miR-155	Correlation with nodal stage and Duke stage	Discrimination of nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages	Yang *et al*[61]
miR-200b-3p	Association with advanced tumor stage and survival	Decreased expression level in advanced tumor stage	Slattery *et al*[59]
miR-215	Association with advanced tumor stage and survival	Decreased expression level in advanced tumor stage	Slattery *et al*[59]
miR-335-5p	Association between any miRNA expression and survival	The expression of miR-335-5p is associated with a better survival	Slattery *et al*[59]
miR-374a	Correlation with metastasis stage	Decreased expression of miR-374a in tumor of patients without metastasis	Xu *et al*[58]
miR-425-5p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-451a	Association with advanced tumor stage	Decreased expression level in advanced tumor stage	Slattery *et al*[59]

CRC: Colorectal carcinoma.

evaluation of response before surgery could spar patients with complete response the surgical resection of the rectum (with all the associated disadvantages), but until today there is no validated biomarker for that. Moreover, since patients respond differently to CRT, a biomarker to predict response to neoadjuvant chemoradiotherapy in rectal cancer patients even before CRT could spar the non-responders the CRT. Understandably, there is a great interest to use miRNAs as possible biomarkers to predict therapy response. Some studies analyzed descriptively the changes in miRNA expression after chemoradiotherapy while others were able to identify miRNAs in tumor tissue, which seem to predict the response to therapy.

Svoboda *et al*[63] performed microarray analysis on tumor biopsies of 31 patients with locally advanced rectal cancer before and 2 wk after chemoradiotherapy with capecitabine (a 5-FU prodrug). They found a significant increase of miR-125b and miR-137 expression levels after 2 wk of chemoradiotherapy. Moreover, they also demonstrated that high levels of miR-125b and miR-137 are associated with a worse response to chemotherapy. However, the sample size is quite short (31 patients), and there is an intertumoral variability described, which should not be neglected. Interestingly, the same group investigated in 2012 in a similar setting 20 patients with locally advanced rectal cancer, whose tumors were classified as most sensible (n = 10) or most resistant (n = 10). They used TaqMan Low Density Arrays analysis to quantify 667 human miRNAs in the tumor tissue samples (preoperative biopsies of untreated primary tumors) and found 8 miRNAs to be significantly differently expressed between the responders and non-responders: miR-215, miR-190b and miR-29b-2 were overexpressed in non-responders while let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a were down regulated in non-responders[64]; the previously identified miRNAs miR-125b and miR-137 were not mentioned.

Drebber *et al*[65] did real-time-PCR analysis to identify the expression of miR-21, miR-143 and miR-145 in macrodissected FFPE tumor tissue of 40 patients before and after chemoradiotherapy. They described a significant upregulation of miR-143 and miR-145 in post-therapeutic tumor tissue compared to pre-therapeutic tumor tissue. In addition, they showed a significant correlation between a low miR-145 expression in the post-therapeutic tumor tissue and a worse response to CRT. However, this result does not address the problem to predict therapy response in advance: The low expression of miR-145 was measured in the post-therapeutic tumor tissue. To predict tumor response miRNA profiles in the pre-therapeutic tissue are needed.

More adequate to this purpose, Della Vittoria Scarpati *et al*[66] analyzed miRNA expression by microarray and confirmed by qRT-PCR in primary tumor biopsies of patients with locally advanced rectal cancer who underwent neoadjuvant CRT followed by surgery (n = 38). Eleven miRNAs were significantly upregulated in patients with a complete response (miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909, miR-630, miR-765) and two were downregulated (miR-1274b, miR-720). However, the small cohort of patients' needs additional validation in an independent cohort[66]. Though, none of the mentioned 13 miRNAs was found when Kheirelseid *et al*[67] performed a similar study by using microarray

analysis of 12 FFPE pre-therapeutic tissue samples of rectal cancer to answer to same question by identifying differentially expressed miRNAs. The promising miRNAs in this study were miR-16, miR-590-5p and miR-153 to predict complete *vs* incomplete response and miR-519c-3p and miR-516 to discriminate between good *vs* poor response. Unfortunately, they do not clarify how these miRNAs are altered between the responders and non-responders (downregulated or upregulated).

A possible reason for the different identified miRNAs may be the difference between the tissues used: Della Vittoria Scarpati *et al*[66] used fresh biopsies frozen in liquid nitrogen while Kheirelseid *et al*[67] used FFPE. However, if we act on the assumption that the type of preservation (FFPE, Kryo, *etc.*) differs the miRNA expression, the next question posed would be: What is the preservation effect on miRNA expression and which miRNA expression profile derives from the different tumor characteristics? On the other hand, Hotchi *et al*[68] used also fresh frozen biopsies from 43 rectal cancer patients before starting CRT and did both microarray analysis and RT-PCR of miRNAs concerning response prediction. They found out that miR-223 was higher expressed in tissue from patients with a good response to CRT and declared miR-223 (which is not mentioned by any other study investigating miRNAs in rectal cancer patients for therapy response prediction) as a promising biomarker for the prediction of response to CRT[68]. Other studies found other different miRNAs: Lopes-Ramos found miR-21-5p to be over expressed in tumor biopsies of rectal cancer patients with complete response using fresh biopsies frozen in liquid nitrogen[69], Bhangu *et al*[70] found miR-200c as a possible biomarker to predict CRT response as it shows a significantly reduced expression in non-responders using FFPE material. Figure 2 shows the important miRNAs concerning response to CRT in rectal cancer patients.

In a recent study of our own group, we were able to show with qPCR-analysis a significant decrease of miR-18b and miR-20a during CRT in plasma of patients with a negative nodal stage after CRT (ypN0) compared to those with a positive nodal stage (ypN+). This data presents miR-18b and miR-20a as possible candidates for biomarkers predicting nodal stage after CRT[71]. However, this data requires validation in a lager cohort.

IN VITRO ANALYSES FOR RECTAL CANCER SPECIFIC MIRNAS

Beside the *in vivo* analyses, functional data of specific miRNA that obviously play a role in rectal cancer have been analyzed. One of these is miR-21 that has already been described above. Using tumor biopsies Chang *et al*[72] showed an inverse relationship between miR-21 and programmed cell death protein 4 (PDCD4), a known tumor suppressor[72]. They hypothesized the post-transcriptional modulation of PDCD4 *via* mRNA degradation. These findings were based on data from Asangani *et al*[73], who transfected Colo206f cells with miR-21 and found a significant suppression of PDCD4 proteins *in vitro*.

For miR-182 Amodeo *et al*[74] investigated the effect on thrombospondin-1 (TSP-1), a protein inversely correlated with tumor vascularity and metastasis. In CRC, TSP-1 is shown to be downregulated. After transfection with anti-miR-182, expression level of TSP-1 increased. Hence, the authors concluded that anti-miR-182 could be used to restore TSP-1 expression in CRC to inhibit the angiogenic and invasive events in CRC.

For another rectal cancer associated miRNA, namely miR-455, rapidly accelerated fibrosarcoma (RAF1) seems to be a target gene: In 20 mucosa and 20 CRC biopsies miR-455, miR-484 and miR-101 seem to be down-regulated. An overexpression of miR-455 in SW480 cells showed inhibition of proliferation and invasion. Western Blot analyses showed a downregulation of RAF1 in cells with an overexpression of miR-455, although, on mRNA-level, there was no effect shown[75]. Also the relevance of miRNAs concerning the sensitivity towards CRT could be assessed *in vitro*: Using 12 colorectal cancer cell lines, the miRNA expression profile indicating sensitivity towards an *in vitro* treatment of 5-FU and radiation was established by our own group[76]. These data were validated by the transfection of let7g, miR-132, miR-224 and miR-320a that led to the expected shift of therapy resistance towards sensitivity. For let-7g the higher expression as a good prognostic marker was validated in patient samples.

POLYMORPHISMS IN MIRNAS

Since miRNAs represent one of the important mechanisms of gene expression control, the relevance of polymorphisms concerning miRNAs has been explored in few studies. Naccarati *et al*[77] showed in a case-control study that two single nucleotide polymorphisms within the 3'untranslated regions of target DNA repair genes (nucleotide excision repair genes), hence the miRNA-binding sites, were significantly associated with rectal cancer: rs7356 in RPA2 (predicted binding miRNA: hsa-miR-3149 and hsa-miR-1183) and rs4596 in GTF2H1 (predicted binding miRNA: hsa-miR-518a-5p, hsa-miR-527 and hsa-miR-1205). This study points out that not only the expression levels of miRNAs are relevant, but also their ability to interact with their target gene.

Jang *et al*[78] tried to identify polymorphisms in *miRNA* genes which have a prognostic value in rectal cancer patients and found 196a2C > T (allele of hsa-miR-196a2) polymorphism to be a significant risk factor for the overall survival of rectal cancer patients. The mentioned allele has been reported by other studies to be involved in increased risk of various cancer types[79-81]. Recently, Mao *et al*[82] found miR-146a being decreased in rectal cancer tissue compared to adjacent normal mucosa and they also showed an association between the genetic variant in miR-146a, rs2910164 polymorphism and the risk of CRC.

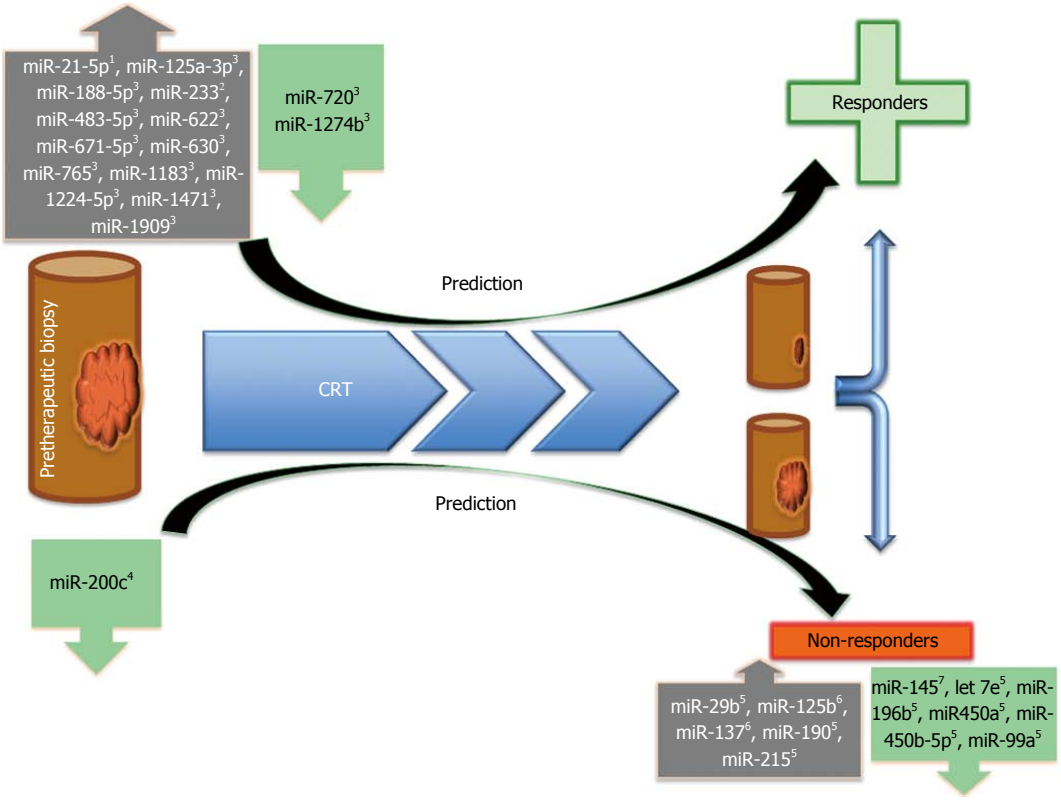


Figure 2 Differential expression of miRNAs dependent on response to preoperative chemoradiotherapy. miRNAs in up arrow callouts are significantly higher expressed; those in down arrow callouts are significantly lower expressed. On the left site there are miRNAs, isolated from pretherapeutic biopsies, which are supposed to predict response or non-response, respectively. The miRNAs in the bottom localized on the right side, are found to be significantly higher or lower expressed in post-therapeutic tumor biopsies of non-responders after chemoradiotherapy compared to pretherapeutic biopsies. [1]Lopes-Ramos *et al*[69], 2014; [2]Hotchi *et al*[68], 2013; [3]Della Vittoria Scarpati *et al*[66], 2012; [4]Bhangu *et al*[70], 2014; [5]Svoboda *et al*[64], 2012; [6]Svoboda *et al*[63], 2008; [7]Drebber *et al*[65], 2011.

CONCLUSION

miRNAs are widely accepted to play a crucial role in physiological and pathological processes. Interestingly, in contrast to the relevance of rectal cancer and its frequency, especially compared to colon cancer, the number of available studies is rather small. The amount of studies as well as the small number of patients per study may be one of the reasons why only few overlapping miRNAs have been identified. Importantly, a small number of miRNAs were identified with relevance in rectal cancer. Many of these are rather known from cancer specific mechanisms than display rectal cancer specificity. Accordingly, the relevance of miRNAs as a predictive or prognostic biomarker in rectal cancer is questionable. Furthermore, the relevance of functional miRNAs does not appear to be as obvious as in previous studies that are typically cell-line based. However, before ignoring the relevance of miRNAs it should be taken into account that human cancer tissue is functionally a rather complex cell system. The analyses are impeded by the heterogeneity of tumor biopsies that in general include different amounts of non-tumor cells such as stroma or the surrounding tissue. Different analyzing techniques applied to identify miRNA (PCR, microarray, *etc*.) or the varying fixation media (FFPE, fresh frozen biopsies, *etc*.) further complicate the comparability of the data. Furthermore, subtle expression differences of a given miRNA that potentially change complex regulatory mechanism simply may not be identified. This may due to the techniques applied or has simply not been part of the analyses that, in general, focus on expression fold changes.

Specifically for miRNAs, there may be alternative reasons for varying results, such as the highly variability of miRNA expression due to external influences such as nutrition. Humphreys for example, showed that the expression of oncogenic miRNAs can be altered by dietary manipulation: A high red meat intake leads to elevated miR-17-92 (cluster) and miR-21 in rectal mucosa tissue of healthy volunteers. While organ specificity is well known for miRNA, Li *et al*[37] identified miRNA expression differences (*e.g*., miR-182) in CRC between African and Caucasian Americans. Possibly, there are further influences like medications used by the patients, gender differences, or age associated variations that are much higher than currently expected.

Overall, there is a large number of possible reasons as to why a clear identification of miRNAs still failed. However, compared to alternative molecular markers in rectal cancer such as proteins, mRNA or DNA, miRNA are not inferior as there are currently no well established markers. Acknowledging some of the previously listed points, miRNA analyses in rectal cancer aiming to identify regulatory mechanisms or to establish marker for

prediction or prognosis should be endorsed. Furthermore, these efforts should be expanded to blood samples as it has been done in many other cancer types.

FUTURE PERSPECTIVES

Validity of cell free and cellular miRNAs as a prognostic or diagnostic tool remains, at least in parts, elusive. The incomplete understanding of biological processes yielding circulating RNAs and their physiological relevance needs to be addressed in more detail, *e.g.*, by application of less bias-sensitive technologies and combinations of, *e.g.*, high-throughput sequencing, qPCR and microarray techniques[83]. Functional characterization of altered miRNAs in CRC and surrounding healthy tissue with respect to more recent findings of modifications that impact miRNA processing and target-gene regulation will improve quality and interpretability of the datasets originating from quantitative analysis[84]. Investigations on differential or coherent expression of miRNAs in affected tissues, changes of strand-selection during tumor progression, and treatment as well as in-deep analyses of the physiological relevance of secreted miRNAs and other non-protein coding RNAs can clarify roles of these and feasibilities to choose particular candidates as markers for prognosis and diagnostics or candidates for therapies[23,36].

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