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Abstract. *Background/Aim: miRNA expression patterns vary within primary rectal cancers and play a pivotal role in carcinogenesis. It is unknown, however, if these regulatory changes also play a role in local recurrent rectal cancers. In this study, the expression of various angiogenetic small non-coding ribonucleic acids, namely miRNA-21, miRNA-215, miRNA-221, and miRNA-222 were analysed in cancerous and healthy rectal tissues. Patients and Methods: miRNA expression was analyzed via quantitative polymerase chain reaction (qPCR). Samples were obtained from 20 patients who were treated for local recurrent rectal cancer at the Department for general and visceral surgery, Klinikum Oldenburg, Germany. Results: No significant differences in the expression of miRNA-221, miRNA-222 and miRNA-215 were observed between cancerous and healthy rectal tissues. However, a significant differential expression was detected for miRNA-21. Conclusion: miRNA-21 is differentially expressed in recurrent rectal cancer tissue and healthy tissues. However, miRNA-215, miRNA-221 and miRNA-222 are not significantly differentially expressed.*

Colorectal cancer (CRC) is the third most common cancer worldwide and the second most common cause for cancer related death (1). In the last decades mortality from rectal cancer declined, however, incidence has been on the rise (2). The prognosis for CRC depends on tumor size, lymphatic and distant metastasis. While the differentiation between colon cancer and rectal cancer is mainly anatomical, several studies have proven major differences on a molecular and genetic level (3-6). Therefore, it is useful to examine each cancer individually. Local recurrence can occur within both entities; however, due to its narrow topography in the lesser pelvis, research has been focused on recurrent rectal cancer.

In addition to the above-mentioned histopathological factors, several different biological markers regarding colon and rectal cancer were identified. These markers were linked to the prognosis of CRC, which led to the establishment of targeted cancer therapy in recent years.

Immunohistopathological as well as matrix-changing mechanisms play a role in the genesis of CRCs. The regulation is based on various genetic transcripts, which in return can be targeted by cancer-specific treatment modalities. Among these regulatory factors, microRNAs (miRNAs) play a role in tumorigenesis and tumor growth. miRNAs are small, non-coding RNA molecules, which are involved in various physiological and pathological processes. They play an important role in fundamental processes such as proliferation, differentiation, apoptosis and angiogenesis. Many miRNA genes lie within cancer-associated genomic locations. Mutations within these loci can lead to changes in the expression profiles of the associated miRNAs. Therefore, it seems reasonable to hypothesize that miRNAs play a crucial role in various phases and processes of malignant diseases (4, 5). Various studies have shown regulatory changes and/or expressions in genomic miRNAs for different malignancies (7-10). Because of their stability within the post-transcriptional process, miRNAs are promising candidates as novel markers in the diagnosis, prognosis and therapy of cancer. The described stability in miRNA stands out compared to other types of RNA (*e.g.* mRNA, tRNA). In addition, miRNAs

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Key Words: Locoregional recurrent rectal cancer, microRNA, angiogenesis, expression, colorectal cancer.

Table I. *Association of miRNA and their clinical parameters regarding primary rectal cancer.*

miRNA	Target gene	Regulation	Clinical effect
miRNA-21	*PDCD-4*; *CDC25a*; *MSH-2*; *MSH-6*	Up-regulation	Worse prognosis, associated with lymphatic metastasis
miRNA-215	Thymidilatsynthase; Dihydrofolatreduktase	Down-regulation	Decreased survival rate, associated with advanced tumor stage
miRNA-221 und -222	c-kit; stst5a; *ETS1*; *RECK*; *NFKB*	Up-regulation	Associated with lymphatic metastasis and tumor stage

appear in almost all body fluids and are not influenced by external environmental factors (6, 11). These characteristics make miRNA an interesting agent to be examined. In previous studies, various miRNAs have been examined and validated to play a role in primary CRCs (12-15). However, their role in locoregional recurrent rectal cancers has not yet been evaluated. The biological behavior of CRCs depends on immunomodulatory, angiogenetic and matrix-regulated processes (16).Various genetic transcripts with regards to their association with established prognostic factors were already examined and validated by various study groups (17).

Recent studies suggest a prominent role of miRNA-21, -215, -221 and -222 in the pathogenesis of cancer. miRNA-21 expression is implicated in the process of intravasation. Particularly, it correlates with invasiveness and metastasis. Additionally, miRNA 21 seems to enhance lymphatic metastasis. The underlying mechanisms is presumably coupled to the tumor suppressor gene Pdcd4 (12, 18).

Expression of miRNA-215 is significantly suppressed in CRC. Intriguingly, the expression of miRNA-215 has been inversely correlated with the expression of thymidylate synthase and dihydrofolate reductase, which was associated with reduced patient survival (19).

MiRNA-221 and -222 play a central role in angiogenesis (20) *via* the inhibition of RNA translation of c-kit, stat5a and ETS1 (16, 21, 22) (Table I). Various studies have shown that expression of miRNA-211 and -222 was significantly higher in CRC and their expression was correlated with the clinical and pathological tumor stage (23).

Changes in the expression profiles of various miRNA sequences in primary CRC have been reported. Here, the angiogenesis of tumors plays a crucial role as this process is targeted by current therapies (16, 24). However, none of these changes have been examined and properly validated for locoregional recurrences. It is unknown whether the reported differences in primary CRC are also relevant for recurrent rectal cancer.

Patients and Methods

Samples from cancerous and healthy rectal tissues were taken from 20 patients with a locoregional recurrent rectal cancer. The cancerous part was taken from the malignancy, while the healthy tissue was taken from the tumor-free tissue margin of the same specimen. RNA of formalin fixed paraffin embedded tissue (FFPE) was isolated with miRNeasy FFPE Kit (Qiagen, Venlo, Netherlands) as previously described (25-27). The quality and quantity of the isolated RNA were assessed by a Tape Station (Agilent, Santa Clara, CA, USA). The translation of RNA into complementary DNA (cDNA) and subsequent qPCR was performed using the miScript II RT and miScript SYBR Green PCR Kit (Qiagen) according to manufacturer's recommendations. Two miRNAs (miR-16 and miR-345) that are stably expressed in CRCs were used as endogenous controls and for normalization (18). ΔΔCT-Method was used for the normalization of the samples (28). RNA from two adenocarcinoma-cell lines (SW-480 and SW-620) served as positive controls in the qPCR reaction.

Statistics. A paired *t*-test was used to determine whether the expression levels of various miRNAs were different in cancerous tissues compared to healthy tissues. Based on various previous studies, we expected a medium effect size (Cohen's d) of d=0.9. Twenty samples were necessary for a power of 0.8 and a *p*-value of 0.05. This study was carried out in accordance with the declaration of Helsinki in its current version. In addition, ethics approval was received from the ethics committee of the Carl-von-Ossietzky University Oldenburg, Germany.

Results

All samples of the cancerous (T) and healthy tissues (N) were examined. N=18 patients already deceased, n=2 are still alive. These two patients gave their permission to analyze the tissue. We were unable to show a significant difference in the expression of miRNA-221, miRNA-222 and miRNA-215. However, an increased expression of miRNA-21 was shown in the cancerous tissue compared to the healthy tissue (Figure 1).

Discussion

MiRNAs have been a focus of research in the past few years due to their promising role in several different diseases, including cancer. Several studies have investigated the role of miRNA in CRC (15, 29-32). However, to our knowledge and available literature, no study has examined the expression of miRNAs in recurrent CRC.

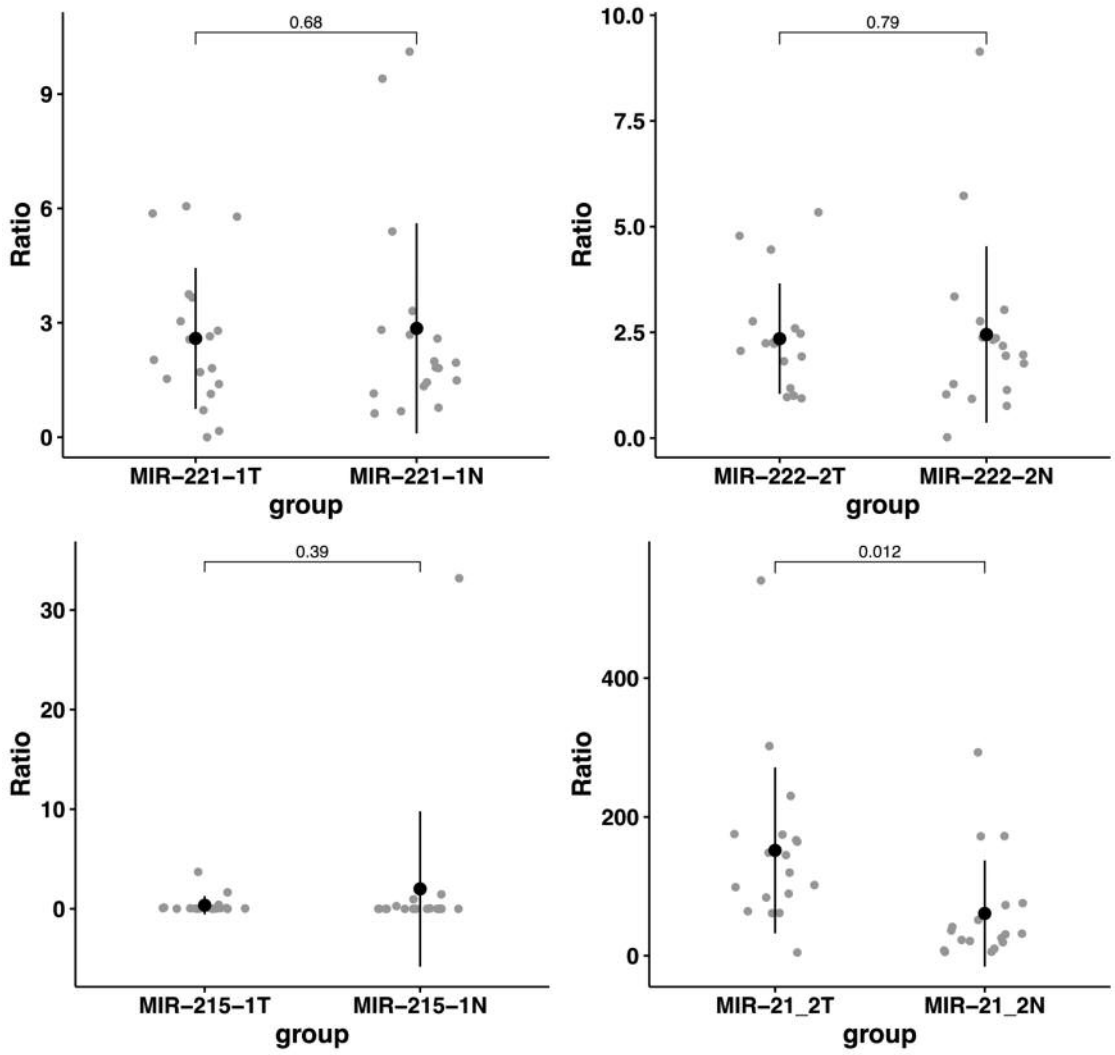

Figure 1. *Comparison of miRNA expression between cancerous (T) and healthy (N) tissues (n=20). Paired t-test of the examined miRNAs (221, 222, 215 and 21). Significant difference is only seen for miRNA-21.*

Our study found an increased expression of miRNA-21 in the cancerous tissue compared to the healthy rectal tissue (Figure 1). The role of miRNA-21 in cancer has been extensively researched (29, 33-35) and several studies also examined the expression and possible roles of miRNA 21 in CRC (12, 13, 30, 36). Rokkas *et al*. (36) have found that miRNA-21, is a promising diagnostic biomarker in CRC. In addition, serum miRNA-21 3p is a promising marker (as part of 5 miRNA panel) to distinguish CRC from healthy patients and those with colorectal adenomas (30). Orosz *et al*. (48) compared the expression of circulating miRNA in the serum and found differences between colonic and rectal cancer patients.

In our study, we did not find any a significant difference in the expression of miRNA-221, miRNA-222 and miRNA-215 (Figure 1). While some studies have found a difference in the expression of these miRNAs between cancerous tissues and the healthy tissues (14, 37, 38), none has examined the expression of these miRNAs in recurrent CRC tissue. Our results could be explained by the differences in miRNA expression between primary CRC and recurrent CRC tissue. However, our study was influenced by several factors that might have contributed to low quality of miRNA in the sample or the expression of these miRNAs.

A review of miRNA expression profiles in rectal cancer has found little overlap in the results of several different studies (39). Technical limitations such as harvesting the tissue samples, storage of tissue samples and technique of measuring miRNA may influence the results. These methodological/technical difficulties have been described as

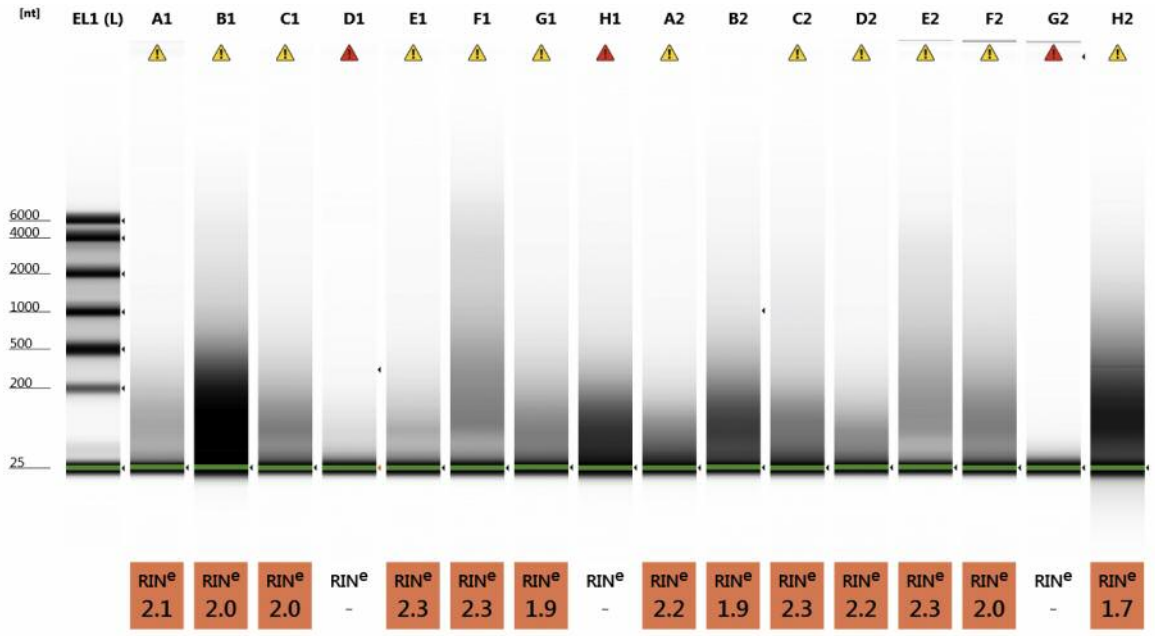

Figure 2. *Examination of RNA quality using the Agilent Tape Station. Low RIN-values (<5) suggest strong degradation of RNA. The analysis of RNA from cancerous and healthy tissue from 8 out of 20 patients is depicted.*

factors as why certain miRNAs could not be repeatedly verified as potential markers (17, 40).

All of our 20 patients received radiochemotherapy before surgery for either the primary rectal cancer or the recurrent rectal cancer. Some studies have described changes in the expression profiles after radiochemotherapy. In some instances, a correlation between miRNA expression patterns and response to radiochemotherapy has been observed (41, 42). Several studies have found that decreased expression of miRNA-21was associated with good response to radiochemotherapy in patients with CRC (42-44). The post-therapeutic changes in the expression levels could be a potential source of error when comparing different data sets (*i.e.* comparing previous results to our own results).

FFPE samples are not the ideal material for molecular and biological analysis. Although FFPE samples can be safely stored at room temperature, the RNA can be degraded none the less. In addition, chemical modifications of the RNA (cross-linking with adjacent molecules) can hamper downstream analysis. Different approaches have been suggested to improve the RNA isolation despite poor RNA quality. After isolation with the miRNeasy FFPE Kit of Qiagen the RNA yield was low. About 10 cuts per sample can yield 50-200 ng of total RNA to be translated into cDNA. Principally, this is sufficient for a cDNA synthesis with subsequent qPCR. Analysis with Agilent Tape Station showed a strong degradation (Figure 2), which was expected due to the long storage of the tissue samples (up to 8 years). The strong degradation of the samples made further analysis difficult. Due to the resulted limitation regarding the RNA isolation, a transcriptome analysis could not be realized. It was anticipated, that RNA isolation out of single cells after laser capture microdissection would not yield the desired results. Therefore, the total RNA was isolated out of 10 cuts and cDNA was synthesized.

In vitro data have shown that single miRNA expression profiles can predict a positive response to, for instance, 5-FU therapy. Additionally, a modification of that expression profile raised the sensitivity of 5-FU and improved the overall prognosis (45). Since the identification of miRNA as a potential regulator of gene expression, the mechanisms of polymorphism have been examined in several studies. Polymorphisms of the target DNA as well as the miRNA have been identified. Moreover, one study has shown that it influences the incidence of rectal cancer (46). Also, a direct correlation between the overall survival of the patients has been shown for the polymorphism of the miRNA-192 (47). Our analysis, however, did not consider possible polymorphisms. This again can be seen as a possible source of error in the evaluation of our data.

Conclusion

In summary, the role of miRNAs in the regulation of genetic expression has been well established. Its role in carcinogenesis, however, remains unclear. Studies that

analyzed patients with rectal cancer are relatively rare and have often a small sample size. This could explain the aforementioned lack of reproducibility of the results. Previous findings from cancer cell lines cannot always be validated *in vivo*. The effects of external factors such as nutrition and harvesting of tissue samples are only partially known and are not always considered/relevant in previous studies.

Our results can partially support our hypothesis. However, it shows that miRNA-21 also plays a central role in locoregional recurrent rectal cancer. To what extent future technologies and/or combination of technologies such as Nano string analysis or microarray technique will improve data quality remains to be seen (28, 29).

The relevance of locoregional recurrent rectal cancer needs to remain a subject of future and subsequent research. The personalization of possible therapies must be the aim to improve overall patient survival and quality of life.

Conflicts of Interest

The Author(s) indicate no potential conflicts of interest related to this study.

Authors' Contributions

NK performed the experiments and wrote and reviewed the manuscript; NES revised the manuscript; UR designed the experiments; HRR performed clinical work; MB reviewed the manuscript; HM designed the experiments and wrote the paper; AT conceived and designed the study and wrote the paper.

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