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Mir-486-5p Downregulation Marks an Early Event in Colorectal Carcinogenesis

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Abstract

BACKGROUND: microRNAs are dysregulated in colorectal cancer and subsets correlated with advanced tumor stage and metastasis. Data are lacking on microRNA dysregulation from early to late stage disease.

OBJECTIVE: Identify a microRNA signature associated with the primary tumor and metastatic site in stage IV disease. Examine whether the signature is evident in earlier stages.

DESIGN: A microRNA profile was generated, then explored in normal colon tissue (N = 5), early stage (stage I & II, N = 10), and late stage (stage III & IV, N = 14) colorectal primary tumors via polymerase chain reaction to delineate molecular events that may promote colorectal carcinogenesis.

SETTINGS: Genome-wide microRNA expression profiling was performed on fourteen patient-matched stage IV primary colorectal cancer tumors and corresponding liver metastases.

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MAIN OUTCOME MEASURES: MicroRNA array technology was used to identify miR expression predictive metastatic potential in the primary tumor.

RESULTS: A distinct nine-member signature group of microRNAs were concurrent in stage IV primary colorectal cancer, and their corresponding liver metastases, when compared to surrounding unaffected colon and liver tissue: microRNA-18b, microRNA-93, microRNA-182, microRNA-183, microRNA21, microRNA-486–5p, microRNA-500a, microRNA-552, and microRNA-941. Of the microRNA panel, only microRNA486–5p was differentially expressed in early stage colorectal cancer samples compared to normal tissue ($p = 0.001$) and additionally differentially expressed between late stage colorectal cancer samples and normal tissue ($p < 0.01$).

LIMITATIONS: Our microRNA profile was generated in a small subset of patients and will require validation in more samples.

CONCLUSIONS: We identified a distinct microRNA signature in primary colon and matched metastatic disease. Upon further investigation, one microRNA was differentially expressed in both early and late stage cancer patient samples and it may herald an early event in colorectal carcinogenesis. This study warrants further investigation with a larger patient cohort to better understand the effect of microRNAs in carcinogenesis. See **Video Abstract** at <http://links.lww.com/DCR/Axxx>.

Keywords

Colorectal carcinogenesis; Early and late stages of disease; Metastasis; microRNA; MicroRNA dysregulation pathways

Colorectal cancer (CRC) remains a leading cause of cancer-related death, despite major advances in early detection and treatment, primarily due to metastatic spread of disease.¹ While sequential mucosal changes, characterized by the accumulation of well-known genetic and epigenetic mutations, lead to development of a primary CRC,^{2,3} the progression to metastatic disease is less well-understood. Twenty percent of patients present with metastatic disease (stage IV), and nearly half of all patients diagnosed with stage II and III tumors will develop metastases.⁴ Currently, adjuvant chemotherapy is standard for patients with lymph node involvement (stage III), but adjuvant chemotherapy for early stage (I or II) disease is not routine, and stage II patients are selected for chemotherapy based on “high-risk” clinical and pathologic features.⁵ The fact that 20% of stage II patients die of recurrent disease⁶ suggests that undetectable pathologic differences exist in tumors that develop metastases. Despite years of gene expression profiling, development of prognostic biomarkers that predict metastatic potential remains elusive.⁷

microRNAs (miRs) have emerged as valuable biomarkers in cancer, as they concurrently target multiple genes in diverse signaling pathways. These small, non-coding RNA molecules function in regulating gene expression,⁸ and have been found to be dysregulated in CRC, specifically in the progression of disease.⁹ Abnormal expression of discrete subsets of miRs (e.g. miR-21, miR-181a, miR-200) correlate with advanced tumor stage and metastasis in CRC.^{10–13} Yet, there is no defined miR signature that will consistently predict early and late events in CRC.^{7,13,14} We posit that a miR expression profile characteristic of metastatic disease may be utilized to identify a progression of events from early stage to late

stage CRC. Our study aims were to identify a miR signature in metastatic CRC that is associated with the primary tumor and its metastatic site. In addition, we aimed to explore their prognostic potential in a cohort of patients with both earlier and later cancer stages compared to normal tissue and identify whether those differential miRs marked an early or late event in the development of carcinogenesis.

METHODS

Patient Selection

Approval from the Institutional Review Board (#4099) was obtained prior to conducting review of archived tissue or patient data. Patients with CRC diagnosis at Oregon Health & Science University (OHSU) and registered in the colorectal tumor registry in conjunction with the OHSU Knight Cancer Institute biorepository were studied. Clinical, pathologic, and outcome data was obtained from medical records, including age at diagnosis, gender, primary tumor location, AJCC stage, pre- and post-surgical chemotherapy, and radiation treatment. Clinical stage for patients with rectal cancer and pathological stage for patients with colon cancer were classified according to AJCC 6th edition staging manual. Corresponding archived formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples of patients diagnosed with colorectal cancer were obtained. All tissue samples were selected by our collaborating pathologist for tumor (>50%) and necrosis percentage (<20%).

MicroRNA Array

Fourteen patients were diagnosed with colorectal cancer from 2006 to 2012, and were treated with both colon and liver resections at OHSU. All but one patient was diagnosed with metastatic disease at initial diagnosis (i.e. synchronous diagnosis).

RNA was isolated from FFPE primary colon tumor and liver metastases patient slides, using RNeasy FFPE Kit (Qiagen catalog # 73504), according to the manufacturer's protocols. The RNA isolation was performed by a single technician to reduce inter-operator variation. Following isolation, RNA was submitted to the Gene Profiling Shared Resource for RNA quality assessment, and microarray assay.

RNA assessment was performed, using the Agilent 2100 Bioanalyzer, and both a Nano 6000 and Small RNA Analysis kit. RNA concentration and purity were determined by UV absorption, using a Nanodrop 1000 spectrophotometer. Quality assessment was conducted on a total of 48 RNAs. RNA samples of acceptable quality and concentration were labeled. The RNA samples examined had obtained a minimum 260/230 value of 1.0, and a quantity minimum of 50ng/μl. The majority of the samples had a quantity over 50 ng/μl, and a quality of 260/230 value closer to 2.0. Genome-wide miRNA expression profiling was performed, using Affymetrix miRNA v. 3.0 Array on 42 samples obtained from 14 patients. The samples were FFPE samples of paired colon tumor, surrounding normal colon tissue, metastatic liver tumor, and surrounding normal liver tissue. There were seven complete quadruplets (tumor colon, normal colon, tumor liver, normal liver) and seven additional pairs of colon and liver tumor samples. The analysis included 3,391 miRNA probes (1,733

miRNA and 1,658 pre-miRNA probe sets), and excluded other small molecule RNAs (scaRNA, snoRNA, CDbox and HACabox), as well as non-human miRNA probe sets.

Raw data files were processed for initial data quality control, and poor performing array assays were eliminated from further analysis. The final data set and downstream statistical analyses included 42 arrays. Array data were preprocessed and normalized using the Robust Multi-array Average (RMA) method, PM (perfect match) background correction, and Quantile Normalization, using the Partek Genomics Suite 6.5 software. Signal intensities were log2 transformed, and probe set values were summarized, using the Median Polish Summarization Method.

Data visualization tools (e.g., box plot, hierarchical clustering, and multi-dimensional scaling) were used to assess the general data quality and outliers. To account for within-subject correlations, the mixed effects model was fit to compare four groups for each probe set, assuming a completely unstructured covariance matrix. The four groups were: Tumor Colon (TC), Normal Colon (NC), Tumor Liver (TL), and Normal Liver (NL). The mixed effects model included all available data (n=42 observations per probe set), accounted within-subject correlations, and performed various comparisons within the same model framework. The following contrast statements were generated to perform specific comparisons: 1) TC vs. NC; 2) TC vs. TL, and; 3) TL vs. NL. P-values from each comparison were adjusted, using the False Discovery Rate (FDR).¹⁵ Probes with FDR p-values of 0.10 or less were considered differentially expressed.

MicroRNA Profile Exploration

For the miR profile exploration, tissue samples of normal colon tissue (N = 5), early stage (Stage I & II, N = 10), and late stage (Stage III & IV, N = 14) colorectal primary tumors were utilized for analysis. As above, RNA was isolated from the FFPE patient tissue sample slides, using the RNeasy FFPE Kit, and the RNA isolation was performed by a single technician to reduce inter-operator variation. RNA was similarly submitted to the OHSU Gene Profiling Shared Resource for RNA quality assessment and quantitation. RNA fragment size distribution below the minimum threshold recommended for sequencing library preparation was excluded.

cDNA synthesis was then performed, utilizing the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cat#4368814), using the Taqman Small RNA Assay Protocol (with a specific oligo for each microRNA). Sequences of miR primers used are listed in **Supplementary Table 1**. qRT-PCR was performed with the Taqman Gene Expression Assays (Applied Biosystems), using the Applied Biosystems ViiA7 qRT-PCR machine. MicroRNA values were normalized to the housekeeping control RNU48.

Target RNA Exploration

Potential targets of the differentially expressed miRs were identified, utilizing OncomiRDB, after a thorough review of publically available miR databases.¹⁶ This database was chosen due to wider profile of miRs. OncomiRDB identified OLMF4 as a downstream target of miR 486-5p. Protein isolation from paraffin fixed tissues was limited, due to the thickness of the

thin tissue sections, and this compromised protein quality due to fixation. Therefore, RNA was isolated from patient samples and qRT-PCR analysis was performed. OLFM4 expression was explored in the early and late stage CRC samples, utilizing qRT-PCR OLFM4 primers (F:TTTCCAATTTACCGGCTCC; R:GCTGTGAATTTCAAGCGTTCC).

Basic descriptive statistics were used to describe patient population of the exploration samples. Relative miR expression was reported, utilizing the comparative CT method as previously published.¹⁷ Characteristics and the distributional properties of the transformed expression values were evaluated for outliers. One outlier was identified and removed. The final sample included the eight-member signature group of miRs from 29 patients. Tests for differences in the miR expression values between groups were conducted, using the Exact Wilcoxon Rank-Sum test with the Bonferroni correction for multiple comparisons. Significance for multiple comparisons was set at $\alpha = 0.016$. For all other tests, significance was set at $\alpha = 0.05$.

RESULTS

A distinct miR signature common in stage IV patient-matched primary CRCs and liver metastases relative to surrounding normal tissue was identified. Patient demographics and treatment factors for tissue used for microarray are described in **Table 1**. A cohort of 64 miRs was differentially expressed in primary CRC tissue, compared to normal colon tissue. There were an additional 170 miRs differentially expressed in the liver metastatic tissue compared to normal liver tissue. Among the 64 CRC miRs and 170 liver miRs, there were nine miRs which were commonly expressed in CRC primary tumors and liver metastases (**Figure 1**). A distinct nine-member signature group of miRs were identified as differentially expressed in both stage IV primary CRC, and their corresponding liver metastases relative to surrounding unaffected tissue: miR-18b, miR-93, miR-182, miR-183, miR-21, miR-486-5p, miR-500a, miR-552, and miR-941 shown in **Table 2** (a heat map analysis is shown in **Supplementary Figure 1**).

Analysis of the distinct miR profile was performed in a population consisting of normal tissue, early stage CRC (stage I&II), and late stage CRC (stage III&IV). Expression was compared in late versus early stage primary CRC tumors, with normal tissue used as the control. Demographic variables, tumor-specific, and treatment factors for the validation samples are summarized in **Table 3**. In this exploration cohort, miR-486-5p was differentially expressed in early stage CRC, compared to normal tissue ($p = 0.001$). miR-486-5p was also differentially expressed between late-stage CRC and normal tissue ($p < 0.01$) (**Figure 2**). The other eight miRs were not differentially expressed between early and late stage CRC (**Table 4**). An AUC was calculated, suggesting that miR-486-5p is highly predictive of early and late stage CRC (AUC=0.98 early vs normal, AUC=0.93 late vs normal).

Pathway analysis was performed, using OncomiRDB. This database identified 151 possible downstream targets of mir-486-5p. Of those targets, OLFM4 was a promising downstream target, based on prior studies discussed later. We isolated RNA and performed qRT-PCR

from patient samples. The target gene OLFM4 was decreased in late stage CRC relative to early stage disease (**Figure 3**).

DISCUSSION

We have identified a distinct miR signature in patient-matched primary site colorectal cancer and metastatic disease that may regulate biologic pathways consistent with an early event in carcinogenesis. In our exploration of this distinct miR signature in early and late stage CRC disease, compared to normal samples, one miR was differentially expressed in early stage CRC compared to normal tissue, and late stage compared to normal tissue: miR-486-5p ($p < 0.01$). Our data suggest that differential expression of this miR in newly diagnosed cancer may herald an early event in colorectal carcinogenesis regulating progression of disease.⁹

Bartley and colleagues were the first to describe the phenomenon of early and late patterns of differential miR expression in the adenoma–adenocarcinoma sequence. Early patterns of altered miR expression were noted when significant changes occurred at the first step in the sequence of disease, that is, between normal mucosa and low-grade dysplasia in adenomas. The second major pattern in miRs was not present in low-grade dysplasia.⁹ They did not explore early versus late stage adenocarcinoma miR dysregulation differences. In contrast, we explored advanced stage miR patterns. We found miR-486-5p is a herald of early events of expression in colorectal carcinogenesis.

In other cancers, miR-486-5p is associated with attenuated tumor growth and lymphangiogenesis.^{18–20} In gastric cancer, specifically, miR486-5p has been closely studied. In a comparison of gastric carcinoma and normal gastric tissues, miR-486-5p was downregulated in gastric cancer cells, similar to our findings in CRC. Using bioinformatics analysis, anti-apoptotic glycoprotein *OLFM4* was identified as a probable target of miR-486-5p, which was subsequently proven in an *in vitro* model. When the gene was silenced, proliferation of gastric carcinoma cells *in vitro* was significantly reduced. This suggests that *OLFM4* may be a downstream target of miR-486-5p in CRC.²¹ In our exploratory analysis of *OLFM4*, contrary to gastric cancer studies, we found decreased expression of *OLMF4* in late stage disease, but increased in early stage disease. This finding may suggest early changes in *OLMF4* expression may lead to advanced disease.

Other biologic functions of miR-486-5P have been proposed. One study miR-486-5p targeted neuropilin-2, a gene involved in cardiovascular development.²² Others have identified its close association with p53, suggesting its role as a tumor suppressor miR in colorectal cell line SW40.²³ A similar study assessed miR expression Caco-2 and HCT-116 cell lines. They found a significant increase in expression in miR-486-5p in K-Ras mutated lines, which was confirmed in 25 cancer patient samples. This association likely needs further assessment, as they did not compare these tissues to normal samples.²⁴ In contrast, we explored a total of 43 patient samples, including five normal and 38 early and late stage disease samples. We observed a decrease in miR-486-5p expression in early and late stage disease, when compared to normal samples. Therefore, when decreased in primary tumors, as in our populous, cell progression may be unchecked.

Upon further exploration of our metastatic miR signature, the remaining miRs were not significantly different; we did, however, note trends in miR-18b, miR-500a, and miR-93. miR-18b has previously been identified in a miR signature that may predict relapse of CRC.²⁵ Its mechanism is unknown, but had been proposed to be a tumor suppressor in melanoma.²⁶ miR-500a was decreased in our early stage cohort, when compared to normal tissue; there is no understanding, however, of its role in tumorigenesis. miR-93 was downregulated in early stage disease. miR-93 expression appears to have a role in the inhibition of colorectal cancer, most notably through the suppression of the Wnt/ β -catenin signaling pathway,²⁷ and the inhibition of genes that promote cell growth, migration, proliferation, and angiogenesis.²⁸ We may not have seen a significant change in these miRs due to our small cohort; they do, however, deserve further study.

Our results need to be carefully interpreted, as there are small changes in the miR expression in our exploration cohort that may be resolved with a greater number of patients. Additional studies involving a larger array of tissue samples will improve our miR stratification of early and late CRC carcinogenesis. A larger array will elucidate miR expression in the presence and absence of neoadjuvant chemoradiation and differentiate tumor location. Although this study employs a small cohort of patients, the findings suggest certain miRs are involved in early patterns of disease progression. Further work is needed to solidify the role of our miRs in conjunction with messenger RNA on carcinogenesis pathways, including OLFM4. Our goal is to continue future studies with a larger cohort of patients with a hope to guide therapeutic efforts at specific events in early carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. miR expression in colon tumor and liver tumor in stage IV CRC patients

Figure 2. Box plots for the delta CT of miR-486–5p expression in normal tissue (control), early stage CRC tissue and late stage CRC tissue

Figure 3. Box plots for the delta CT of OLFM4 expression in early stage CRC tissue and late stage CRC tissue compared with normal

Table 1.
Characteristic of Patient Samples used for Microarray Analysis

Stage IV Patient Samples for microarray (N = 14)	
Age (median at diagnosis)	51.5
Gender (% male)	64%
Primary Cancer Site	11 - Colon 3 - Rectal
Timing of metastatic diagnosis	11 - Synchronous to CRC primary 2 - At time of initial surgery 1 - Metachronous to CRC primary
Neoadjuvant CRT	1 - CRT 7 - Chemo only 4 - No neoadj tx 2 - Unknown
Adjuvant CRT	11 (78.6%)
Follow-up (median in years)	3 yrs
Clinical status	10 - Alive 2 - Deceased 2 - Unknown

Table 2.
miR array analysis reveals potential biomarker “fingerprint” in Stage IV disease when compared to normal tissue.

miR Probe	Primary Colorectal Cancer (Fold Change)	Colorectal Metastases Liver (Fold Change)
miR-182	6.59	20.49
miR-18b	3.29	4.43
miR-552	3.27	7.14
miR-183	3.21	5.36
miR-500a	1.74	4.63
miR-93	1.54	3.40
miR-941	1.54	1.40
miR-21	1.51	1.63
miR-486-5p	-7.26	-3.82

Differentially expressed miR signature in primary CRC and metastases relative to normal control tissue. Significance = $p < 0.01$

Table 3.
Patient demographics in the exploration profile of early and late stage CRC patients.

	Early Stage CRC Patients N = 10 N (%)	Late Stage CRC Patients N = 14 N (%)
Patient Demographic Factors		
Age of diagnosis (median)	64 years	61 years
Gender		
Male	6 (60%)	10 (71%)
Tumor Specific Factors		
Tumor location		
Colon	6 (60%)	10 (79%)
Rectum	4 (40%)	3 (22%)
Tumor grade		
Well differentiated	1 (10%)	1 (7%)
Moderately differentiated	9 (90%)	10 (71%)
Poorly differentiated		3 (21%)
AJCC-6 Stage^a		
Stage I	5 (50%)	
Stage II	5 (50%)	
Stage III		9 (64%)
Stage IV		5 (36%)
Treatment Factors		
Cancer Directed Surgery		
Yes	100%	100%
Neoadjuvant Therapy		
Chemotherapy only	N = 1 0 (0%)	N = 4 2 (15%)
Combined CRT	1 (10%)	2 (17%)
Adjuvant Therapy^b		
Chemotherapy only	N = 1 1 (9%)	N = 12 11 (85%)
Combined CRT	0 (0%)	1 (8%)
Follow Up Data		
Median Follow-Up (years)	6.5 years	2.7 years
Recurrences/Progression		
Distant metastases	3 (30%)	1 (8%)
Locoregional recurrence	3 (30%)	0 (0%)
No evidence of disease	4 (40%)	9 (64%)
Never disease-free/death	0 (0%)	4 (29%)

CRT = Chemotherapy and radiation therapy

Table 4.
Comparisons in the Mean MicroRNA Expression Values for Normal Tissue, Early and Late Stage Colorectal Cancer

miRNA	Mean Delta Ct (Sth Dev) by group			Wilcoxon rank sum p-value:		
	Normal	Early	Late	Normal	Normal	Early
				vs	vs	vs
	N = 5	N = 10	N = 14	Early*	Late	Late*
18b	0.0013 (0.0007)	0.0006 (0.0008)	0.0006 (0.0007)	0.055	0.056	0.886
93	0.6201 (0.2186)	0.3000 (0.3801)	0.4081 (0.3602)	0.028	0.107	0.172
182	0.0079 (0.0053)	0.0112 (0.0153)	0.0134 (0.0101)	0.768	0.298	0.213
183	0.0036 (0.0026)	0.0068 (0.0141)	0.0158 (0.0182)	0.371	0.186	0.559
486-5p	0.0814 (0.0301)	0.0175 (0.0132)	0.0243 (0.0314)	0.001	0.003	0.666
500a	0.0107 (0.0017)	0.0066 (0.0065)	0.0092 (0.0089)	0.040	0.343	0.546
552	0.0053 (0.0037)	0.0132 (0.0254)	0.0094 (0.0089)	0.594	0.444	0.508
941	0.0256 (0.0161)	0.0085 (0.0089)	0.0090 (0.0104)	0.055	0.044	1.000

Note. Differences in the mean miRNA $2^{-\Delta\Delta C_t}$ between groups was tested with the Exact Wilcoxon Rank-Sum Test. Significance was adjusted for multiple comparisons using the Bonferroni correction ($k=3$, $p = 0.016$). Comparisons that are significantly different from zero are denoted in bold and italics.