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Complementary Strand MicroRNAs Mediate Acquisition of Metastatic Potential in Colonic Adenocarcinoma

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Abstract

BACKGROUND and AIMS—Altered expression of specific microRNAs (miRNA) is known to occur during colorectal carcinogenesis. However, little is known about the genome-wide alterations in miRNA expression during the neoplastic progression of primary colorectal cancers.

METHODS—Using a miRNA array platform, we simultaneously evaluated the expression of 668 miRNA in primary colonic adenocarcinomas. Prediction analysis for microarrays (PAM) was used to identify differentially expressed miRNA. Biological functions of selected miRNA were evaluated with *in vitro* invasion assays.

RESULTS—Primary fresh frozen tissues from 65 patients (40 male and 25 female) with a mean age 65 $\pm$ 13 years and with AJCC Stages I (n=7), II (n=22), III (n=18) and IV (n=18) colon cancers, underwent RNA extraction and miRNA array analysis. We identified a seven-miRNA expression signature that differentiated Stage I and Stage IV primary colon cancers. We then demonstrated this signature was able to discriminate between Stage II and III primary colon cancers. Interestingly 6 of the 7 differentially expressed miRNA were downregulated in association with the development of metastases, and all 7 miRNA were complementary strand

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miRNA*. We transfected HCT-116, a highly invasive colon cancer cell line, with corresponding downregulated miRNA* and demonstrated that overexpression of 3 of the 6 miRNA* (miR200c*, miR143*, and miR424*) significantly abrogated invasive potential.

CONCLUSION—We have identified a seven-miRNA* signature that is associated with metastatic potential in the primary tumor. Forced over-expression of 3 of the 6 identified miRNA* resulted in an attenuation of *in vitro* invasion, suggesting direct tumor suppressive function and further supporting the biological importance of complementary strand miRNA*.

Keywords

Metastases; Complimentary strand MicroRNA; Colon Cancer

INTRODUCTION

Colorectal cancer is the second most common cause of cancer mortality in western societies, accounting for approximately 51,000 deaths yearly in the United States alone¹. The vast majority of these deaths are secondary to the sequelae of metastatic disease, which is reflected in current staging algorithms. For example, the presence of lymphatic metastases (AJCC stage III disease) or distant metastases (AJCC stage IV disease) corresponds to an anticipated five-year survival rate of 66% and 10% respectively, as compared to 90% for patients with early stage cancers. Although the association between mortality and metastases is clear, the genomic mechanisms underlying tumor cell dissemination and the acquisition of metastatic potential remain poorly understood. However, cellular regulation of a relatively new class of small RNAs, termed microRNAs (miRNA), may play an integral role in this malignant process^{2–6}.

miRNA are small (~22bp) endogenous RNAs that are known to serve important regulatory functions in many cellular processes including development, differentiation, proliferation and cell death⁷. miRNA are initially transcribed by RNA polymerase and processed in the nucleus by DROSHA to form a pre-miRNA, which consists of a hairpin loop of RNA containing the miRNA and a complimentary miRNA (denoted with *). Following nuclear export, pre-miRNA are processed by DICER to yield both the mature miRNA and a mature complimentary strand miRNA* (the latter of which has until recently^{8–10} thought to be degraded as a non-functional by-product of miRNA metabolism secondary to decreased stability). miRNA are subsequently loaded into the RISC complex, where they are then able to alter the cellular transcriptome through translational inhibition and/or mRNA degradation.

A single miRNA can regulate hundreds of genes, and it has been estimated that 30% of all genes are regulated by miRNA. Given that differential regulation of a single miRNA can have broad cellular consequences, miRNA have been postulated to potentially serve as a superior class of cancer biomarkers; prognostic gene signatures may be created with relatively small numbers¹¹. Additionally, several miRNA have previously been demonstrated to play regulatory roles in mediating colorectal cancer cell metastases¹². Nonetheless, few studies have utilized comprehensive miRNA profiling to study colorectal cancer. We sought to evaluate miRNA expression in colon cancer across the spectrum of disease, focusing on

the identification and evaluation of miRNA that may serve both predictive and functional roles during metastatic progression.

METHODS

Tissue Samples

Tissues from patients undergoing surgical resection for colorectal cancer were collected with IRB approval, and stored in the Tissue Procurement Core. Histopathological examination of resected specimens (histological type, tumor size etc.) was performed by an experienced GI pathologist (D.C.). A representative section of tissue was cut and stained with hematoxylin and eosin to confirm the diagnosis. Macrodissection of formalin fixed paraffin-embedded (FFPE) tissues was performed to isolate tumor sections (containing >90% tumor cells) for RNA extraction. Tumors that occurred in the background of genetic cancer syndromes or in the presence of inflammatory bowel disease were excluded.

MicroRNA Isolation and Labeling

Total RNA was isolated using the *mirVANA*TM miRNA Isolation Kit (Ambion Inc.) according to the manufacturer's protocol, including enrichment for small RNAs. RNA concentration and quality was determined using NanoDrop1000 (Thermo Fisher Scientific). Only RNA samples of sufficient quality were used. MicroRNA expression profiling was performed utilizing the TaqMan® Array Human MicroRNA Panel v1.0 (Early Access) (Applied Biosystems), a microfluidic card that contains 668 miRNA assays and five small nuclear RNAs (snRNA) that function as endogenous controls for data normalization. Single stranded cDNA was synthesized from 200 ng of total RNA in eight Multiplex RT primer pool reactions containing stem-looped RT primers that were only specific to the mature miRNA. Quantitative PCR was carried out under the following thermocycler conditions: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. The resulting eight cDNA samples were diluted, combined with TaqMan Universal PCR Master Mix (Applied Biosystems), and then loaded onto a TaqMan Low Density MicroArray Panel, allowing for the simultaneous quantification of all 668 human miRNAs and 5 endogenous controls.

Statistical Analysis

miRNA signature development—Our overall algorithm for generation and evaluation of a miRNA signature is shown in Figure 1. Prediction analysis for microarrays¹³ (PAM) was used to compare AJCC stage I versus stage IV primary colon cancer and build a miRNA signature. Cross-validation was used to evaluate performance of prediction error rate. A miRNA set with the smallest overall error rate was selected as our miRNA signature. False discovery rate (FDR) was controlled at 1%.

Derivation of miRNA signature—In accordance with our previously published work¹⁴, an overall miRNA signature was generated using the first principal component to represent the overall expression level for our miRNA signature. The miRNA signature was then applied in discriminating AJCC Stage II versus Stage III primary colon cancers.

Data normalization—Raw Ct values above 32 were considered undetectable (i.e., no expression). RNU44 was used as the endogenous control gene to calculate average delta-Ct. Delta-Ct values were converted to the log scale by subtraction of the maximum of delta-Ct value to each delta-Ct value (i.e., miRNA expression value=maximum (delta-Ct) - delta-Ct). The converted miRNA expression value was used for statistical analysis (Figure 1; data are available in GSE29622).

Evaluation of Histopathologic Association—We assessed the prognostic potential of the miRNA signature by examining its association with tumor grade/stage using the Pearson correlation and with overall survival using log-rank test based on the median cutoff.

Cell Culture and Transfection

HCT-116 cells were maintained in RPMI medium 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, in a humidified incubator containing 5% CO₂ at 37°C. Twenty-four hours prior to transfection, HCT-116 cells were seeded in 6-well plates at 2×10^5 cells per well and incubated overnight at 37°C. Transfection was performed with Lipofectamine-2000 (Invitrogen) and 50 ng/well of pre-miRNA for the following miRNA: miR-200c*, -15b*, -424*, 143*, -378*, -106b*, and negative control (Applied Biosystems), according to the manufacturer's protocol.

Quantitative Real-Time PCR

Successful pre-miRNA transfection and subsequent miRNA overexpression in HCT-116 was confirmed using quantitative real-time PCR. Briefly, reverse transcription was carried out with TaqMan MicroRNA Assay Kit (Applied Biosystems) using miRNA*-specific primers. Quantitative real-time PCR for our miRNA and an endogenous control, RNU44, was performed using TaqMan Universal PCR Master Mix on a 7500 Fast Real-Time PCR System according to the manufacturer's protocol. miRNA expression levels were quantified and relative miRNA expression was calculated by comparative cycle threshold method (2^{-CT} method), with RNU44 as the endogenous control.

Invasion Assay

Invasive potential was measured by the cells' ability to compromise an extracellular matrix in the presence of a chemo-attractant using ECM pre-coated transwell membrane inserts (CytoSelect™ 24-well Cell Invasion Assay, Cell Biolabs Inc). HCT-116 cells were harvested 48 hr after transfection and transferred to serum-free RPMI medium. Cell suspension was added to the upper chamber, and the lower chamber was filled with 10% FBS which functioned as a chemo-attractant. Following 24 hr incubation at 37°C, non-migrated cells were removed with cotton swabs. Cells that invaded the lower surface of the membrane were stained with Dapi (company), and counted per 100 high-power fields with an inverted microscope. All experiments were run in duplicate and performed on two separate occasions.

RESULTS

Clinicopathologic Data

Primary tumor specimens from 65 patients with colon cancer (40 male, 25 female) of mean age of 65 ± 13 years were evaluated. Tumor-specific pathologic data are depicted in Table 1.

Differentially Expressed miRNA

A total of 7 miRNA demonstrated significant differential expression between primary AJCC Stage I tumors and primary Stage IV tumors using PAM at threshold of 2.5 and controlling the FDR to $<1\%$. The overall error rate was 8% by cross-validation. Decreased expression was observed for 6 miRNA, while over-expression was observed for a single miRNA in comparing primary AJCC Stage I and IV tumors (Table 2). The aggregate miRNA signature was able to significantly differentiate between primary AJCC Stage I and IV tumors ($p=0.0002$; Table 2). The aggregate miRNA signature was also able to significantly differentiate between primary AJCC Stage II and III tumors ($p=0.008$; Table 2). In evaluating each miRNA individually in primary AJCC Stage II tumors versus primary Stage III tumors, 4 miRNA demonstrated significantly decreased expression (Table 2). Of note, the miRNA signature was unable to differentiate AJCC stage I and stage II primary tumors (neither of which are associated with metastases) or AJCC stage III and stage IV primary tumors (associated with lymph node and distant metastases, respectively). Interestingly, we also evaluated each corresponding non-complimentary miRNA of each miRNA* in our signature and found no similar correlations. We also failed to identify any consistent relationship between the level of expression of any individual miRNA* in our signature and its corresponding non-complimentary miRNA (Figure 2) (i.e. overexpression of miRNAs* were not consistently associated with co-overexpression or down-regulation of the corresponding miRNA).

miRNA Signature and Tumor Grade Correlation

As expected, given our use of stage to derive differentially expressed miRNA, our miRNA signature showed association with overall survival ($p=0.007$). Interestingly, however, our miRNA signature was associated with decreasing tumor differentiation from well to poorly differentiated ($r=-0.38$ with $p=0.002$; Table 3, Figure 3). The Pearson correlation was -0.67 ($p<0.0001$) when the grade/stage were ranked from well-differentiated of stage I to poorly differentiated of stage IV.

miRNA Transfection and Invasion

Given that 6 of the 7 miRNA of interest were down-regulated with tumor metastases, we sought to evaluate their ability to abrogate *in vitro* invasion through forced over-expression. We measured the endogenous expression of the 6 miRNA in HCT-116, a highly invasive colon cancer cell line, and found negligibly low expression for each miRNA (Ct value range 32–38). Prior to evaluation of invasive potential, we confirmed successful transfection with qRT-PCR for each miRNA of interest. After transfection, the mean expression Ct value was 17 (range 16.5–17.5) (Figure 4). Invasion was significantly suppressed with the over-expression of 3 miRNA; miR-200c*, miR-143*, and miR-424* (Figure 4) as compared to

cells transfected with the negative miRNA control. Additionally, we simultaneously overexpressed these three miRNA (miR-200c*, miR-143*, and miR-424*), and demonstrated the greatest degree of abrogation of metastatic potential, greater than that seen with the overexpression of any single miRNA* (Figure 4).

DISCUSSION

Although the molecular alterations leading to tumor cell acquisition of metastatic capability remain unknown, miRNA have recently been implicated as serving an important role through post-transcriptional regulation^{REF}. We therefore evaluated the expression of 668 miRNA primary colon cancers across the spectrum of stages using high-throughput miRNA technology. We identified a seven-miRNA signature, composed entirely of complementary strands, which correlated with tumor metastases. Interestingly, six of the seven miRNA were down-regulated with AJCC stage progression, suggesting tumor suppressor function. Additionally, forced over-expression of 3 miRNA resulted in attenuation of *in vitro* invasion, providing further evidence of direct tumor suppressive function, and supporting the biologic importance of complementary strand miRNA.

Prior to fulfilling their regulatory roles through association with argonaute (AGO) proteins, miRNA exist in a miRNA:miRNA* duplex. Although the ultimate fate of the miRNA* strand was thought to be linked to the sorting pathways that act on their partner miRNA, such that loading of the two strands was inextricably linked in a ying-yang fashion, recent evidence suggests otherwise. In *Drosophila*, miRNA* species require canonical RNAi factors for their accumulation similar to endogenous siRNA and ultimately associate with AGO2, whereas miRNA associate with AGO1 through thermodynamic asymmetry¹⁰. Similarly, Ghildiyal et al. demonstrated that loading of miRNA* in AGO2 is dependant on a unique combination of canonical miRNA and siRNA factors in *Drosophila*⁹. These data indicate independent regulation of partner miRNA:miRNA*, unveiling a new layer of complexity in miRNA metabolism. We found no relationship between the measurable expression of our seven miRNA* and their partner miRNA, which is similarly suggestive of independent mechanisms of regulation for miRNA and miRNA* in higher organisms.

The traditional view of tumor metastases involving a step-wise accumulation of advantageous mutations is changing. The importance of epigenetic regulation, and in particular alterations in miRNA expression, has come to the forefront of metastases research^{15–18}. Prerequisite accumulation of multiple genetic changes has been obviated by data demonstrating that multiple phenotypes can be acquired through a single alteration. For example, differential regulation of a single miRNA, miR-31, has been shown to modulate multiple phenotypes including motility and invasion in breast cancer cells¹⁹. Similarly, we have identified a panel a metastases-associated miRNA in colonic tumors, and demonstrated the ability of a subset of the panel to abrogate *in vitro* invasion in colon cancer cells. Our panel is however composed entirely of miRNA*, which have only recently been demonstrated to play a functional role in tumor biology.

miRNAs* have demonstrated *in vitro* tumor suppressive regulation of important oncogenic genes. Tsang and Kwok, through forced over-expression and repression using multiple

cancer cell lines, have shown that miR-18a* negatively regulates K-Ras, with a demonstrable impact upon cell proliferation and anchorage-independent growth²⁰. Kim et al. similarly demonstrated the pro-apoptotic effects of miR-199a* through forced overexpression in fibroblasts as a direct result of down-regulation of the MET proto-oncogene and its downstream effector ERK2²¹. miRNA over-expression experiments however need to be interpreted with caution. Competition is known to exist among small RNA for argonaute protection, and saturation of argonaute binding capacity through introduction of exogenous miRNA has been shown to limit the function and stability of endogenous miRNA with unexpected but profound biological consequences^{22–26}. Although we have not yet identified the gene targets of miR200c*, miR143* or miR424*, it is important to note that the phenotypic changes we observed with forced over-expression are not simply a consequence of miRNA regulatory perturbation- we utilized identical transfection conditions as well as pre-miRNA concentrations for all experiments and identified biological significance with over-expression of 3 of 6 down-regulated miRNA.

Curiously, miR200c has been widely associated with development of the epithelial-to-mesenchymal (EMT) phenotype which is characterized by an invasive tumor cell with metastatic potential, drug resistance, and stem cell like properties^{27–32}. We have recently demonstrated that an EMT signature is intrinsic to a large population of human colorectal cancers and that this signature is closely linked to the expression of miR200c and its inhibition of ZEB1 and ZEB2³³. Here, we report the intriguing result that the complementary strand miR200c* is associated with poor prognosis and metastatic development, and therefore may also be linked to the now recognized phenotype of EMT.

In addition to *in vitro* functional studies, miRNA* have recently been evaluated in patient tumor samples. Liu et. al. identified miR-23b* over-expression in 70% of renal cell carcinoma samples as compared to corresponding normal kidney, with a concomitant reduction in protein expression of the novel tumor suppressor proline oxidase³⁴. Jazdzewski et al demonstrated that a SNP in pre-miR146a led to the generation of two distinct complimentary strand miRNA, miR-146a*G and miR-146a*C, which had a profound impact upon host cell transcriptomes and led to a predisposition toward the development of papillary thyroid carcinoma³⁵. Additionally, Schulte identified differential expression of several miRNA* when comparing favorable versus unfavorable neuroblastoma³⁶. We identified the differential regulation of 7 miRNA* in primary colon cancers, none of which have been previously reported, although clearly the evaluation of complimentary strand miRNA is in its infancy. In addition to our results and those reported in renal, thyroid and neural malignancies, a comprehensive bioinformatic and microarray analysis has shown that miRNA* impact vertebrate regulatory networks and likely play significant roles during oncogenesis³⁷, which in aggregate may serve as a catalyst for increasing investigation of the role of miRNA* in various cancers.

We derived a metastatic miRNA signature from primary non-metastatic colon cancers under the premise that progressive identifiable alterations in miRNA expression occur until such a time that a threshold is reached, prompting acute acquisition of metastatic capability and cellular dissemination from the primary tumor. We believe this threshold is breached by a minority population of cells within the primary tumor, although some degree of progression

toward the threshold is reflected in the bulk of the tumor mass. If conceptualized in the context of an epithelial-to-mesenchymal transition (EMT)-like process, we have potentially identified a panel of synergistic pro-epithelial miRNA whose expression levels inversely correlate with tumor dedifferentiation (Figure 3) and metastatic potential. Our signature may therefore be useful in stratifying early stage (AJCC I and II) primary colon cancers for the potential of associated occult metastatic disease and thereby identification of patients that may benefit from early aggressive adjuvant therapy.

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Algorithm

Figure 1.
Algorithm for generation and evaluation of a metastases associated miRNA signature.

Figure 2.
Relationship Between Complimentary Strand miRNA* and Corresponding Non-Complimentary Strand miRNA.

Figure 3.
Correlation between miRNA signature and tumor grade.

Figure 4.
miRNA overexpression in HCT-116 affects *in vitro* invasive capacity.

Table 1.

Tumor Specific Histopathological Data

	Stage I	Stage II	Stage III	Stage IV
“T” Stage	T2 (n=7)	T3 (n=19) T4 (n=3)	T2 (n=1) T3 (n=17)	T3 (n=16) T4 (n=2)
“N” Stage	N0 (n=7)	N0 (n=21)	N1 (N=15) N2 (N=3)	N0 (n=4) N1 (n=10) N2 (n=4)
“M” Stage	M0 (n=7)	M0 (n=21)	M0 (n=18)	M1 (n=18)
Tumor Grade	well (n=2) mod (n=5)	well (n=1) mod (n=18) poor (n=3)	well (n=1) mod (n=12) poor (n=5)	mod (n=16) poor (n=2)

Table 2.

miRNA Signature Evaluation Across the Spectrum of Primary Colon Cancers

	Regulation	AJCC Stage I vs. IV			AJCC Stage II vs. III			AJCC Stage Correlation (I to IV)	
		Fold Change	p-value [^]	ROC	Fold Change	p-value [^]	ROC	r	p-value ^{^^}
7-microRNA signature		0.05	0.0002	0.98	0.31	0.0081	0.80	−0.63	<0.0001
miR-200c*	Down	0.07	0.0006	0.99	0.77	0.5396	0.53	−0.38	0.0016
miR-15b*	Down	0.02	0.0005	0.96	0.12	0.0049	0.75	−0.59	<0.0001
miR-424*	Down	0.08	0.0042	0.91	0.45	0.0779	0.67	−0.53	<0.0001
miR-143*	Down	0.05	0.0220	0.84	0.33	0.1347	0.63	−0.48	0.0001
miR-135a*	Up	11.05	0.0037	0.94	2.06	0.1263	0.66	0.49	0.0001
miR-378*	Down	0.10	0.0001	0.98	0.16	0.0004	0.84	−0.60	<0.0001
miR-106b*	Down	0.05	0.0001	0.96	0.11	0.0024	0.80	−0.55	<0.0001

[^] two-sample test^{^^} Pearson correlation test

All p values were adjusted by FDR

Table 3.

miRNA Signature Correlation with Tumor Grade

	Regulation	Tumor Grade Correlation (Well to Poor Diff)	
		r	p-value^^
7-microRNA signature		−0.38	0.002
miR-200c*	Down	−0.33	0.0168
miR-15b*	Down	−0.31	0.0176
miR-424*	Down	−0.41	0.0046
miR-143*	Down	−0.24	0.0575
miR-135a*	Up	0.32	0.0170
miR-378*	Down	−0.34	0.0168
miR-106b*	Down	−0.21	0.0895