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miR-143 and miR-145 are down-regulated in ulcerative colitis: putative regulators of inflammation and protooncogenes

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Abstract

Background—miR-143 and miR-145 are believed to function as colon cancer tumor suppressors as they inhibit colon cancer cell growth and are down-regulated in sporadic colonic tumors. We speculated that miR-143 and miR-145 might also be down-regulated and contribute to malignant transformation of colonic epithelium in longstanding ulcerative colitis (UC).

Methods—Biopsies were obtained 20 cm proximal to the anus from individuals with quiescent UC and from normal controls. RNA and proteins were extracted and measured. miR-143 and miR-145 were quantified by real time PCR and miR-145 was also assessed by in situ hybridization. Putative targets of these miRNAs, K-RAS, API5, MEK-2 (miR-143), and IRS-1 (miR-145) were determined by Western blotting. To assess the effects of miR-143 and miR-145 on these predicted targets, HCT116 and HCA-7 colorectal cancer cells were transfected with miR-143 and miR-145 and expression levels of these proteins were measured.

Results—In UC, miR-143 and miR-145 were significantly down-regulated 8.3-fold (3.4–20.1) ($p < 0.0001$) and 4.3-fold (2.3–7.8) ($p < 0.0001$), respectively, compared to normal colon. In contrast, IRS-1, K-RAS, API5, and MEK-2 were up-regulated in ulcerative colitis consistent with their assignments as targets of these miRNAs. Furthermore, transfected miR-143 and miR-145 significantly down-regulated these proteins in HCT116 or HCA7 cells.

Conclusion—Compared to normal colonic mucosa, in chronic ulcerative colitis miR-143 and miR-145 were significantly down-regulated and their predicted targets, IRS-1, K-RAS, API5, and MEK-2 were up-regulated. We postulate that loss of these tumor suppressor miRNAs predispose to chronic inflammation and neoplastic progression in IBD.

Background

Patients with ulcerative colitis (UC) are at increased risk for colorectal cancer (CRC) (1, 2). Although the estimated likelihood of CRC in UC varied substantially in previous reports, a meta-analysis published in 2001, which included 116 studies, calculated the cumulative CRC risk as 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease (1). The risk of inflammatory bowel disease (IBD)-associated colon cancer is related to disease extent and duration, as well as severity of inflammation (3–5). With chronic inflammation,

the epithelium undergoes recurrent injury and healing that is thought to predispose to the risk of malignant transformation (6, 7). Molecular and genetic changes accompany this transformation and may occur before there is histological evidence of dysplasia (6, 8–11). In this regard, several previous studies have reported differences in colonic mucosal gene expression in IBD patients compared to normal controls (12–15).

microRNAs (miRNAs) are a recently described class of non-coding RNAs that regulate gene expression. This regulation is mediated predominantly by binding to the 3' untranslated region of messenger RNA (mRNA), resulting in translational inhibition or destabilization of mRNA (16). miRNAs also control normal stem cell fate and differentiation. Supporting their postulated pathogenic roles, many miRNAs are dysregulated in inflammation and carcinogenesis with altered expression profiles in chronic inflammatory conditions and neoplasms of the colon (17–21)

Polycistronic miR-143 and miR-145 on chromosome 5 are down-regulated in sporadic colon cancer (22, 23). A recent report also found that loss of miR-145 induces pro-inflammatory signals in the innate immune system (22–24). While not commented upon, loss of miR-143 was even greater than loss of miR-145 in that study (24). Two previous studies have examined the expression of miRNAs in small cohorts of patients with ulcerative colitis, but alterations in miR-143 and miR-145 were not reported in these studies (21, 25). Patients who were enrolled in these studies, however, did not all have longstanding disease (>7 years). Since miR-143 and miR-145 are expressed in normal colonocytes, down-regulated early in colon cancer, and appear to have a role in regulating inflammation, it was of interest to examine their expression in chronic UC, an inflammatory condition associated with increased risk of colonic neoplasia.

As miR-143 and miR-145 regulate several genes implicated in colon cancer progression, investigation of their targets in chronic UC might provide biologically relevant insights into cancer development. Previous reports have confirmed or suggested several important downstream targets of these miRNAs including API5, cdk6, cyclin D2, E-cadherin, ERK5, IRS-1, K-RAS, and MEK-2 (22, 26–29). Because epithelial cell turnover is increased in UC and UC-associated dysplasia and believed to increase the risk of malignant transformation by locking in mutations, we focused on four predicted targets that are involved in cell cycle regulation, proliferation, and apoptosis: IRS-1 (miR-143), K-RAS, API5, and MEK-2 (miR-145) (30–33).

Methods

Patients

The study was approved by the institutional review board at the University of Chicago. Healthy controls and individuals with chronic UC from our tertiary IBD center were included in the study. Informed consent was obtained from individuals prior to undergoing colonoscopies that were performed as part of their indicated clinical care. Two cohorts, including both healthy controls and UC individuals, were analyzed in the study. Real time PCR was performed on samples from cohort 1. Western blots and *in situ* hybridization were performed using samples from cohort 2. This strategy of using two cohorts was adopted to maximize RNA and protein extractions. In protocols that yield both RNA and protein on the same sample we have found yields of each were lower. In both cohorts, individuals were included if they were ≥ 21 years of age with no personal or first degree family history of colorectal cancer, or personal history of advanced neoplasia (prior polyp > 1cm size, >3 adenomas, or high grade dysplasia). All individuals with UC had 1) disease duration > 7 years, 2) no history of dysplasia, 3) disease extent ≥ 20 cm proximal to the anus and 4)

quiescent colitis without active inflammation. Criteria 3 and 4 were confirmed by an expert GI pathologist (JH).

Sample Collection

Four standard sized colon biopsies were obtained 20 cm proximal to the anus in patients without endoscopic evidence of active inflammation. The biopsies were flash frozen in liquid nitrogen and stored at -80°C until RNA or protein extraction was performed.

RNA extraction

RNA extraction was performed using the miRCURY™ RNA isolation kit (Exiqon). Frozen tissue was placed in 300 μL lysis solution and homogenized using a mechanical homogenizer. Following homogenization, 600 μL lysis buffer was added to the solution. Proteinase K (20 μL) was then added to the lysate and incubated at 55°C for 15 min. RNA was purified using spin columns per manufacturer's directions with DNAase treatment. RNA concentrations and purity (280 nm/260 nm ratio) were measured with Agilent nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Quantitative Real Time PCR (qPCR)

cDNA was prepared by reverse transcriptase from 100 ng total RNA using specific primers and the SuperScript III Platinum Two-Step qRT-PCR kit (Applied Biosystems). TaqMan quantitative real time polymerase chain reaction (qPCR) was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers and probe for miRNA-143, miRNA-145, and reference gene RNU48 were synthesized by Applied Biosystems. PCR thermal profile included 40 cycles at 95°C for 10 min, 95°C for 15s, followed by 60°C for 30s in the MX4000™ Multiplex Quantitative PCR System (Agilent Technologies, Santa Clara, CA, USA).

Western Blotting

Proteins were extracted in SDS-containing Laemmli buffer and quantified by RC-DC protein assay. Proteins (50 μg) were resolved by SDS-PAGE (4–15% gradient gels). A pre-stained molecular weight marker was included on each gel. Proteins were transferred overnight to immobilon-P membranes. Nonspecific antibody binding was blocked by incubating the blots for 2 hrs at room temperature in Tris buffered saline/0.05% Tween-20, (TBST) containing 5% nonfat dry milk. Membranes were incubated overnight at 4°C in 5% milk or 3% BSA with specific antibodies to API5 (1:500; Sigma-Aldrich, St. Louis, Mo USA), IRS-1 (1:1000; Cell Signaling Technology), K-RAS (1:250; Santa Cruz Biotechnology), and MEK-2 (1:500; Epitomics, Burlingame, CA USA), and followed by 1 hr incubation with appropriate secondary antibodies conjugated to horseradish peroxidase (1:3000). Blots were re-probed for β -actin to confirm comparable protein loads. Blots were washed in TBST and specific proteins detected by xerography on XOMAT™ AR film using an enhanced chemiluminescence system. The xerograms were digitized with an Epson flat bed scanner and quantified using Un-Scan-It Gel 5.3 software (Silk Scientific Corporation, Orem, UT, USA) for data acquisition and analysis.

In Situ Hybridization

In situ hybridization for miR-145 was performed using a 5'-end digoxigenin-labeled Locked Nucleic Acid-modified mirCURY miR-145 detection probe or scrambled LNA oligonucleotide as negative control (Exiqon) as described by Silahtaroglu et al (34). Briefly, 12 micron cryosections from biopsies taken 20 cm proximal to the anus were fixed in 4% paraformaldehyde for 10 min. Sections were treated with an acetylation buffer (500 μL of 6N HCl, 670 μL triethanolamine, 48.5 ml of DEPC treated water, 300 μL acetic anhydride), pre-

hybridized for 30 min at 55°C with 100 µl hybridization mixture (50% formaldehyde, 5 × SCC, 500 µg/µl yeast tRNA, 1 × Denhardt's solution, 1.8 ml DEPC-treated water, 9.2 mM citric acid) and then hybridized with probe (3 pmol in 100 µl hybridization solution) for 60 min at 55°C. After washing with 0.1×SCC at 63°C and blocking with 3% hydrogen peroxide at room temperature, sections were incubated for 30 min in a incubation chamber in blocking buffer [0.1M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% BSA, and 0.5% blocking reagent (Perkin Elmer, Downers Grove, IL, USA)]. Sections were treated with anti-FITC/HRP diluted 1:400 in blocking buffer for 30 min at room temperature. Sections were then placed in the incubation chamber in the dark with a 1:50 dilution of FITC-tyramide in amplification buffer (Exiqon, Vedbae, Denmark). After washing with TNT and drying, each slide was treated with 25µl of ProLong® gold containing 4'5'-diamidino-2-pheylindoline (DAPI). Sections were imaged using an epifluorescence microscope equipped with charge-coupled device camera and image analysis software, and intensity values were recorded in appropriate wavelengths.

Tissue Culture

HCT116 colon cancer cells were obtained from ATCC and HCA-7 colorectal cancer cells were obtained from Susan Kirkland (ICRF, London, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂–95% air as recommended by ATCC. Cells were cultured in McCoy's 5A Modified Medium containing 10% serum and miRNA transfection experiments were conducted on pre-confluent cells. Cells (2×10⁵ cells/well) were seeded in 6 well plates and grown for 24 hrs. Cells were then transfected with control oligonucleotide or 5, 10, 20, or 50 nM of mimics of mature miR-143 or miR-145 (Ambion) and harvested 48 hrs later.

Statistical Analysis

PCR data were analyzed based on the 2^{ΔΔCt} approach (35). For each miRNA, a mixed-effects analysis of variance (ANOVA) model was fitted with Ct as the response variable; disease status (normal vs. UC), gene type (miRNA or RNU48), and disease × gene interaction as the fixed effects; and patient as the random effect (36). A linear contrast was constructed to estimate ΔΔCt and its confidence interval, and the results were exponentiated to obtain the estimate of 2^{ΔΔCt}. Statistical analysis was performed with SAS (Cary, NC). Western Blots were compared by Student's t-test.

Results

Baseline Characteristics

As summarized in Table 1, cohort 1 included 8 healthy controls and 8 individuals with chronic UC. In cohort 1, the mean age and sex distributions were similar between healthy controls and individuals with UC. Seven of the 8 individuals with UC were taking oral mesalamine, and two of the eight UC individuals were on maintenance therapy with immunomodulators. The median disease duration of individuals with UC was 20 years (range 10–40).

Cohort 2 included 11 healthy controls and 11 individuals with chronic quiescent UC. In cohort 2, the median age was also similar between UC and controls (56.8 vs. 60.8 years). The majority of control individuals were women (63%), whereas the majority of UC patients were men (73%). All UC individuals were taking mesalamine, and 55% were on immunomodulator therapy in cohort 2. As in cohort 1, the median disease duration of UC individuals was 20 years (range 8–60).

miR-143 and miR-145 are down-regulated in chronic ulcerative colitis

miR-143 was 8.3-fold (3.4–20.1) lower in UC colonic mucosa compared to normal colonic mucosa ($p < 0.0001$, Fig. 1). Similarly, miR-145 was 4.3-fold (2.3–7.8) lower in UC colonic mucosa compared to normal colonic mucosa ($p < 0.0001$, Fig. 1). As shown in Figure 2 and in agreement with these findings, *in situ* miR-145 staining in epithelial crypts was decreased 5.7-fold in UC compared to normal colon. The expression of miR-145 was confined to the crypt and surface colonic epithelial cells (Fig. 2).

KRAS, IRS1, MEK-2, and API5, putative targets of miR-143 and miR-145, are up-regulated in UC

To begin to assess the biological relevance of down-regulation of these miRNA, we examined normal and UC colonic mucosa for demonstrated targets of these miRNA. We selected IRS-1, K-RAS, MEK-2, and API-5, a subset of confirmed targets of these miRNAs in colon (IRS-1 and K-RAS) or pancreatic cancer cells (API5, MEK-2) (22, 26, 28). These proteins are involved in cell cycle regulation, proliferation, and apoptosis. As crypt cell turnover is higher in UC mucosa and in IBD-associated malignant transformation, we hypothesized that these targets might be up-regulated in patients at increased risk for neoplastic transformation (32, 33, 37). Furthermore, these proteins are predicted or established to play causal roles in colonic tumorigenesis (22, 38–40). We have also shown in separate preliminary studies that these proteins varied inversely with miR-143 and miR-145 that are differentially expressed in normal right (high) and left colon (low). Western blotting expression levels of all four proteins in sigmoid colon were higher in ulcerative colitis ($n=11$) compared to normal controls ($n=11$). Relative expression between UC and normal colon was: 3.4 ± 3.6 for IRS-1, 1.92 ± 0.8 for K-RAS, 1.5 ± 0.7 for MEK2, and 2.2 ± 0.7 for API5. Differences in K-RAS ($p=0.03$) and API5 ($p=0.02$) expression reached statistical significance, whereas IRS-1 and MEK-2 did not (IRS-1, $p=0.13$; MEK-2, $p=0.09$), although there appeared to be a trend (Figure 2). Because the control group had a higher proportion of females than male patients, we compared expression of all proteins between male and female subjects in cohort 2 and did not identify sex-dependent differences.

miR-143 and miR-145 downregulate putative targets in HCA-7 and HCT116 cells

To begin to directly test whether these proteins are targets of miR-143 and miR-145 in colon cancer cells, we examined the effects of transfecting exogenous miRNAs on expression levels of these putative targets. We screened several colon cancer cell lines for basal expression of putative targets of these miRNA. Based on these preliminary experiments, we selected HCA-7 and HCT116 cells for these studies. HCA-7 and HCT116 cells were transfected with mimics of mature miR-143 and miR-145 or scrambled control. We showed that transfected miRNA significantly down-regulated IRS-1 (miR-145), and K-RAS, API5, and MEK-2 (miR-143) in these cells (Figure 3).

Discussion

We demonstrated for the first time that miR-143 and miR-145 are down-regulated in chronic UC compared to normal colonic mucosa. These miRNAs have been reported as down-regulated in colon cancer and inflammation, although this is the first report describing decreased expression of miR-143 and miR-145 in ulcerative colitis (23, 24, 41). Comparing UC and control mucosa, expression levels of K-RAS, MEK-2, API-5, and IRS-1 varied inversely with levels of these miRNAs. These findings are in agreement with *in silico* target prediction models. Since these miRNAs are believed to be tumor suppressors in sporadic colonic carcinogenesis, we speculate that their loss might also contribute to the increased risk of cancer in ulcerative colitis. Previous studies have demonstrated that transfection of miR-143 or miR-145 inhibits cell proliferation.

In this study, we examined expression levels of four putative or demonstrated target proteins of miR-143 and miR-145: IRS-1, K-RAS, MEK-2, and API5. These proteins are involved in cell cycle regulation, proliferation, and apoptosis. As there is increased cell turnover in UC associated with neoplastic transformation and transfection of miR-143 and miR-145 inhibits proliferation in transfected cells, these cell cycle and cell death regulators were of interest to study in high-risk individuals (11, 23, 30–32, 42). This is the first study in ulcerative colitis to determine expression levels of these proteins that have potential roles in inflammation-associated neoplastic transformation. IRS-1 is frequently up-regulated in several types of cancers, including colon cancer (43–46). Conversely, IRS-1 reduction increases apoptosis and inhibits β -catenin-induced neoplastic transformation (45). K-RAS and MEK-2 are upstream activators of ERK that drives proliferative signals in colon cancer (38, 47). Anti-apoptotic protein API5 suppresses E2F-dependent apoptosis and is up-regulated in several cancers (39, 48–50). Thus, each of these proteins has experimental support suggesting they might contribute to colon cancer development. In studies *in vitro*, we confirmed that miR-143 transfection down-regulates K-RAS, MEK-2, and API5, and miR-145 transfection suppresses IRS-1 in colorectal cancer cells (22, 28). This is the first study in colon cancer cells to implicate MEK-2 and API5 as targets of miR-143. Although we were able to demonstrate direct regulation of protein expression by these miRNAs *in vitro*, we cannot confirm this inverse correlation *in vivo* as our study design used separate patient cohorts to assess miRNA and protein levels.

Patients with chronic ulcerative colitis are at increased risk for neoplastic transformation. Molecular and genetic changes can occur even prior to histological changes. These include p53 mutations, microsatellite instability, as well as methylation of CpG islands, (8, 51–55). Similarly, gene expression profiling reveals changes in chronic quiescent ulcerative colitis compared to normal controls (56). Although prior investigations into miRNA expression in UC have focused on inflammation, changes in gene expression in quiescent disease may provide insights into causal events driving cancer development (21, 57). In two prior miRNA analyses in ulcerative colitis, changes in miR-143 and miR-145 were not reported in quiescent UC (21, 25). Potential factors accounting for these discrepancies include differences in patient populations or regional sites sampled and methodological differences such as miRNA array compared to qPCR analyses. In contrast to prior studies, our UC patient cohort was more homogenous with absence of clinical inflammation and disease duration ≥ 7 years that is associated with increased risk of malignant transformation. Additionally, it should be noted that we have extended prior studies by examining predicted targets of miRNAs dysregulated in UC both *in vivo* and *in vitro*. Because we did not include patients with histological evidence of inflammation, we were unable to determine the effects of inflammation or therapy on miR-143 and miR-145 expression in the colon.

In summary, miR-143 and miR-145 are down-regulated in chronic ulcerative colitis. While mechanisms of persistent inflammation and IBD-associated carcinogenesis remain obscure, we speculate that suppression of miR-143 and miR-145 contribute to inflammation and predispose to neoplastic progression. Further studies to identify mechanisms down-regulating these miRNAs could provide new insights into the pathogenesis of inflammation and IBD-associated carcinogenesis and might suggest potential pharmacologic targets for their prevention.

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A.

microRNA	$2^{-\Delta\Delta C_t}$ (95% CI)	P-value
miR-143	0.12 (0.05–0.29)	<.0001
miR-145	0.23 (0.13–0.43)	<.0001

B.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Patient Characteristics

	Cohort 1		Cohort 2	
	Control	UC	Control	UC
N	8	8	11	11
Age (mean)	49.3	51	58.0	60.3
Men:women (%)	75:25	62:38	36:64	73:27
Disease Duration (yr,median)	0	20	0	20
Extent of colitis (% Left-sided/Pancolitis)	0	38:62	0	27:73
Medications (%) [*]				
5-ASA	0	88	0	100
Immunomodulator	0	25	0	63

^{*} No patients were on treatment with anti-TNF agents