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Small RNA sequencing of sessile serrated polyps identifies microRNA profile associated with colon cancer

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Abstract

Sessile serrated adenoma/polyps (SSA/Ps) of the colon account for 20–30% of all colon cancers. Small non-coding RNAs, including microRNAs (miRNAs), may function as oncogenes or tumor suppressor genes involved in cancer development. Small RNA sequencing (RNA-seq) was used to characterize miRNA profiles in SSA/Ps, hyperplastic polyps (HPs), adenomatous polyps and paired uninvolved colon. Our 108 small RNA-seq samples' results were compared to small RNA-seq data from 212 colon cancers from the Cancer Genome Atlas. Twenty-three and six miRNAs were differentially expressed in SSA/Ps compared to paired uninvolved colon and HPs, respectively. Differential expression of *MIR31-5p*, *MIR135B-5p* and *MIR378A-5p* was confirmed by RT-qPCR. SSA/P-specific miRNAs are similarly expressed in colon cancers containing genomic aberrations described in serrated cancers. Correlation of miRNA expression with consensus molecular subtypes suggests more than one subtype is associated with the serrated neoplasia pathway. Canonical pathway analysis suggests many of these miRNAs target growth factor signaling pathways.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords

colon cancer; gene expression; hyperplastic polyps; microRNAs; RNA sequencing; sessile serrated adenoma/polyps

1 | INTRODUCTION

Serrated colon polyps are commonly found during screening colonoscopy and are subdivided into hyperplastic polyps (HPs), sessile serrated adenoma/polyps (SSA/Ps), and traditional serrated adenomas.¹ Traditional serrated adenomas are rare while SSA/Ps and HPs are common. Unlike HPs, SSA/Ps may contribute up to a third of all colon cancers.^{2–4} However, differentiating SSA/Ps from HPs is often difficult in clinical practice, and better markers of SSA/Ps are needed for accurate diagnosis and subsequent cancer prevention strategies.^{5,6} We have previously defined an RNA signature in SSA/Ps that distinguishes SSA/Ps from HPs and identifies a subtype of colon cancer likely to develop from SSA/Ps.^{7,8}

The serrated polyposis syndrome (SPS) is an extreme phenotype that often presents with multiple serrated polyps and carries a high risk (up to 42%) of colon cancer development.^{9–11} In clinical practice patients with SPS are routinely screened with annual colonoscopy, while patients with sporadic SSA/Ps are screened every 3 to 5 years. Unlike familial adenomatous polyposis and Lynch syndrome, SPS lacks a known underlying genetic mutation. SPS is likely influenced by epigenetic mechanisms that do not require mutations in protein-coding genes. Increased methylation of gene promoters at CpG islands is observed in SSA/Ps and the underlying colon mucosa of SPS patients, suggesting that such methylation may contribute to altered gene expression in SSA/Ps.² However, it remains unknown what changes in RNA expression in SSA/Ps are required for progression to cancer.

MicroRNAs (miRNAs) are small non-coding RNAs of 18–24 nucleotides that regulate gene expression through mRNA degradation and translation inhibition.¹² A single miRNA may influence the expression of thousands of genes and multiple cellular processes necessary for the development of colon polyps and cancer.¹³ MiRNA expression may also be regulated by DNA methylation.¹⁴ Studies investigating miRNA-mRNA interactions in colon cancer cell lines have identified gene networks contributing to a malignant phenotype.¹⁵ We have investigated miRNA expression by small RNA sequencing (RNA-seq) in prospectively collected colon polyps and normal colon to identify gene networks important in the serrated pathway to colon cancer.

The few studies describing miRNA expression in serrated colon polyps have used microarray or reverse transcription quantitative PCR (RT-qPCR) with retrospectively collected formalin-fixed paraffin-embedded (FFPE) tissue samples,^{16–18} rather than the much more comprehensive RNA-seq approach used in this study and presented for the first time. Also, no prior studies have examined mRNA targets of miRNAs differentially expressed in SSA/Ps. Our RNA sequencing analysis provides a more sensitive and complete analysis of miRNAs, including the discovery of unannotated, novel small RNAs. The goals of our study are threefold: First, define miRNA signatures for serrated polyp subtypes, SSA/Ps and HPs; second, compare serrated polyp-specific miRNAs with colon cancer

subtype miRNA profiles; and third, identify signaling pathways likely playing a role in SSA/P development. We describe a unique miRNA profile in SSA/Ps that correlates with specific genomic aberrations and consensus molecular subtypes (CMSs) of colon cancer. We identify both growth factor and developmental signaling pathways in SSA/Ps that may drive progression to colon cancer.

2 | MATERIALS AND METHODS

2.1 | Patients

Biopsy samples were obtained from routine screening or surveillance colonoscopy patients between the age of 46 and 80 who visited University of Utah Health Care Hospitals. Patients with SPS were between the age of 25 and 76. Subjects with family history of colon cancer, familial cancers including familial adenomatous polyposis and Lynch syndrome, history of inflammatory bowel disease or prior colonic resections were excluded. Samples for RNA sequencing were prospectively collected from 2011 to 2016. All patients provided informed consent as approved by the University of Utah's Institutional Review Board (IRB).

Ten SSA/Ps with matched uninvolved mucosa and two HPs without matched uninvolved mucosa were obtained from six patients with SPS.⁷ SSA/Ps from five of these patients were previously analyzed by long mRNA sequencing.⁸ Right colon was defined as the colonic region from splenic flexure to cecum. Left colon was defined as descending colon to rectum. Sporadic SSA/Ps ($n = 6$, five right colon, one left colon), HPs ($n = 12$, two right colon, ten left colon), and adenomatous polyps (APs, $n = 14$, ten right colon, four left colon) along with matched uninvolved mucosa ($n = 26$) were collected from screening colonoscopy patients. Normal colon tissue ($n = 30$, 15 right colon, 15 left colon) was obtained from patients with no polyps or other pathology found on current or prior exams. If polyps were found during colonoscopy, biopsies of polyp tissue were collected for both routine histopathological diagnosis and for storage in RNeasy as previously described.⁷ All samples were collected prospectively and placed in RNeasy immediately after removal, stored at 4°C overnight and then at -80°C prior to performing RNA isolation.

2.2 | FFPE tissue samples

Forty-seven FFPE samples, consisting of 26 SSA/Ps and 21 HPs, were obtained from the University of Utah's pathology tissue core. Patients had granted consent under IRB-approved protocols for tissue banking. Samples were collected between years 2012 and 2018.

2.3 | Pathological classification

All biopsy specimens were reviewed by expert gastroenterology pathologists at the University of Utah. Serrated polyps were classified according to the recent recommendations of the Multi-Society Task Force on Colorectal Cancer (CRC) for post-polypectomy surveillance and as described previously.^{7,8,19} None of the SSA/Ps used in this study showed dysplasia.

2.4 | RNA isolation

Total RNA was isolated from colon biospecimens using TRIzol (Thermo Fisher Scientific, Waltham, MA) and the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) according to manufacturer protocol. Briefly, each colon sample (20–50 mg tissue) was homogenized in 1 mL of TRI-zol using a tissue homogenizer (Benchmark), and the homogenate was loaded onto Zymo-Spin IC columns. After on-column DNA digestion with DNase 1, the quantity of the eluted RNA was determined by NanoDrop 1000 (Thermo Fisher Scientific) spectrophotometry. For FFPE samples, ten 10- μ m paraffin sections of each FFPE tissue sample were deparaffinized with Neo-Clear (Fisher Scientific, Hampton, NH) and total RNA isolated using the Roche Diagnostics High Pure miRNA isolation kit.

2.5 | Small RNA sequencing, alignments, and quality control

Small RNA sequencing was performed on 108 individual colon samples: 16 SSA/Ps (10 syndromic and 6 sporadic), 14 HPs, 14 APs, 34 uninvolved colon from the above polyp patients and 30 normal control colonic samples from patients without polyps or other colon pathology. Three small RNA-Seq runs of 11, 54, and 43 samples were performed during the course of the study. PCR-amplified cDNA sequencing libraries were prepared using NEBnext (New England BioLabs, Ipswich, MA) multiplex small RNA library prep (97 samples) or Illumina TruSeq small RNA sample prep (11 samples) according to the manufacturers' protocols. Illumina single-end 50 bp sequence reads were performed with an Illumina HiSeq 2500 instrument. After removal of adapter sequences, sequence reads were aligned to the GRCh38/Hg38 human reference genome using novoalign (Novocraft) or STAR aligners.²⁰ Visualization tracks were prepared for each dataset using the USeqRead Coverage application and viewed using the Integrated Genome Browser as previously described.⁸ Quality control metrics and novel miRNA predictions were performed using the online bioinformatics application Oasis.²¹ All raw and processed RNA-seq data files are available from the Gene Expression Omnibus under accession number GSE118504.

2.6 | Differential expression analysis

2.6.1 | Polyp comparisons to paired uninvolved colon—Differentially expressed small RNAs across polyp subtypes were identified using paired DESeq2 statistics implemented in the online bioinformatics application Oasis.^{21,22} To avoid potential batch effects, each polyp and corresponding paired uninvolved colon were run in the same small RNA-Seq run. Among HPs, 10 of 14 had paired uninvolved tissue available. Paired uninvolved tissue was available and used for all SSA/Ps and APs. The *P* values were controlled for multiple testing using the Benjamini and Hochberg false discovery rate (FDR) method. Hierarchical clustering of differentially expressed genes was performed using Cluster 3.0 and Java Treeview software as previously described.^{7,8}

2.6.2 | Serrated polyp and control colon comparisons—Normalization of small RNA-seq data from SSA/Ps ($n = 16$), HPs ($n = 14$), matched right uninvolved colon ($n = 10$), control right ($n = 15$), and control left colon ($n = 15$) was performed using the voom method as part of the limma package in Bioconductor.²³ Statistical differences in small RNA levels

between SSA/Ps and HPs, uninvolved and control right colon, and control left and right colon were determined using the Lmfit and eBayes applications in limma.²⁴

2.7 | Sensitivity and specificity analysis of MIR31–5p

The sensitivity and specificity of *MIR31-5p* in differentiating SSA/Ps from HPs were determined using Receiver Operating Characteristic (ROC) statistics. The ROC curve was generated using the “roc” function in the “pROC” package of the R statistical computing language, version 3.2.1. The 95% confidence interval for the area under the curve (AUC) was estimated from 1000 bootstrap samples.

2.8 | qPCR validation of differentially expressed miRNAs

RT-qPCR was applied to 84 of the same 108 RNA later-stored colorectal tissues that had been analyzed previously by small RNA-seq. These 84 included 15 SSA/Ps (10 syndromic and 5 sporadic), 10 HPs, 12 adenomatous polyps (APs), 15 uninvolved from patients with SSA/Ps, 6 uninvolved from patients with HPs but not SSAPs or APs, 10 uninvolved from patients with APs but not SSA/Ps, and 16 normal colon samples. cDNA was generated from 7 ng total RNA of each sample with the Taq-Man Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA #A28007). Ten μ L qPCR reactions each included 3 μ L cDNA (diluted 1/12 following the synthesis kit's final step), the TaqMan Advanced miRNA Assay primer set (Applied Biosystems, #A25576) for each miRNA, plus TaqMan Fast Advanced Master Mix (Applied Biosystems, #4444557). PCR reactions were assembled in wells of 384-well MicroAmp Optical reaction plates (Applied Biosystems, #4309849) and run in triplicate in a Life Technologies QuantStudio 12 K Flex real-time PCR instrument using cycling conditions recommended by the manufacturer for that master mix. miRNA levels' fold changes were determined using the $\Delta\Delta$ CT method, and statistical significance determined with the Mann Whitney *U*-test. Let-7g-5p miRNA was chosen as the reference miRNA for our PCRs' relative quantitation based on its moderately high expression level and its lack of shift according to the prior RNA-Seq results across our sample categories. The six miRNAs examined by PCR were as follows (with their Applied Biosystems' Assay ID designations in parentheses): *MIR31-5p* (478015_mir), *MIR21-3p* (477973_mir), *MIR378A-5p* (mmu482643_mir), *MIR151A-3p* (477919_mir), *MIR135B-5p* (478582_mir), and *MIRLET7G-5p* (478580_mir). Two miRNAs, *MIR31*, and *MIR135B*, found differentially expressed between SSA/Ps and HPs, were further examined in a new cohort of 47 FFPE samples (26 SSA/Ps and 21 HPs) using the same RT-qPCR procedure.

2.9 | Comparison to TCGA colon cancer RNA-seq data

We compared our differentially expressed SSA/P miRNAs (FDR < 0.05, Supporting Information Tables S1 and S2) to small RNA-seq colon cancer data publically available in the Cancer Genome Atlas (TCGA).²⁵ Two hundred and twelve TCGA colon cancers had both genomic aberration and CMS data available for our analysis. Of 32 differentially expressed miRNAs, only 24 could be evaluated in TCGA colon cancer data. Publically available TCGA small RNA-seq data were previously aligned to the Hg19 human reference genome and do not contain read data for seven recently identified miRNAs, or miRNAs with symbol numbers above 3000. Therefore, *MIR3141*, *MIR3614*, *MIR3656*, *MIR3960*, *MIR4485*, *MIR4488*, and *MIR6089* were excluded from the analysis. *MIR1299* was also

excluded from the analysis because only 3 of 212 cancers had measurable read counts for this miRNA. The expression of 24 miRNAs (17 differentially expressed to paired uninvolved colon, 6 differentially expressed to HPs and 1 differentially expressed to both) was examined in 212 colon cancer small RNA-seq datasets. We compared the expression of each miRNA according to *BRAF* and *KRAS* mutation status, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and CMS.

2.10 | Pathway analysis of putative mRNA targets

To predict mRNA targets for differentially expressed miRNAs we used the TargetScanHuman prediction tool within the Ingenuity miRNA target filter (Qiagen, Hilden, Germany) application.²⁶ TargetScan predicts biological targets by searching for conserved sites in mRNAs that match the seed region of each miRNA. After identifying putative mRNA targets for each miRNA, we used our previously obtained mRNA sequencing data⁷ to filter for mRNAs with expression that was negatively correlated with miRNA expression. Canonical pathways were determined for all differentially expressed mRNAs ($n = 5029$) and mRNAs negatively correlated ($n = 1390$) with one or more miRNAs differentially expressed in SSA/Ps.

3 | RESULTS

3.1 | Small RNA distribution and abundance

Small RNA sequencing was performed on 108 prospectively obtained colon biospecimens. The mean sequence depth was 5.5 million uniquely mapped reads per sample. There were 1010 unique RefSeq small RNAs expressed in human colon (10 reads in one or more samples), with 95% of those RNAs representing miRNAs (653, 65%) and small nucleolar RNAs (snRNAs, 299, 30%) (Supporting Information Figure S1A). The majority of reads (84%) mapped to annotated miRNAs, with 54% of miRNA reads coming from five miRNAs *MIR192*, *MIR21*, *MIR148A*, *MIR143*, and *MIR200B*. The average read count for each of the 653 miRNAs across all biospecimens is shown in Supporting Information Figure S1B.

3.2 | Differential gene expression analysis

3.2.1 | Polyp comparisons to paired uninvolved colon—Differentially expressed small RNAs in SSA/Ps, HPs, and adenomatous polyps (APs) were determined using paired DESeq2 negative binomial statistics as part of the online Oasis DE analysis module.²¹ We compared 16 SSA/Ps to paired uninvolved colon and identified 23 differentially expressed (Fold 1.5, FDR < 0.05) miRNAs, 5 snRNAs and 1 piRNA (Supporting Information Table S1). Two novel miRNAs, one on chromosome 10 and one on chromosome X, were also differentially expressed in SSA/Ps using a less stringent statistical cutoff (Fold 1.4, FDR < 0.1). In contrast, when comparing 10 HPs to paired uninvolved colon, only one miRNA (*MIR4488*) and one piRNA (*PIR020579*) were differentially expressed (Fold 1.5, FDR < 0.05), and both were also differentially expressed in SSA/Ps. Hierarchical clustering and principal component analysis (PCA) of the 23 differentially expressed miRNAs across the 26 serrated polyps are shown in Figure 1A,B, respectively. Cluster 3 in the heatmap shows eight miRNAs with the largest and most consistent increases in expression in most SSA/Ps but not HPs. Cluster 1 shows a similar pattern for five downregulated miRNAs in most

SSA/Ps compared to HPs. Cluster 2 identifies a subgroup of eight SSA/Ps with high expression of *MIR144*, *MIR451*, and *MIR486*. Cluster 4 shows six miRNAs that are similarly expressed in SSA/Ps and HPs. We also compared 14 APs to paired uninvolved colon and identified 8 differentially expressed (Fold 1.5, FDR < 0.05) miRNAs and 1 piRNA (PIR012734) (Supporting Information Table S1). Three of the eight differentially expressed miRNAs in APs were also differentially expressed in SSA/Ps. The fold change and significance of the most differentially expressed miRNAs (Fold 2, FDR < 0.05) across the three polyp types are shown in Table 1.

3.2.2 | Serrated polyp comparisons—Following voom normalization and lmfit and eBayes statistical analyses, we identified six miRNAs differentially expressed (Fold 1.5, FDR < 0.05) between 16 SSA/Ps and 14 HPs (Supporting Information Table S2). Similar to comparisons to paired uninvolved colon, *MIR31-5p* was the most differentially expressed miRNA (11-fold, FDR < 0.05) in SSA/Ps compared to HPs (Figure 2). One other miRNA (*MIR615-3p*) was overexpressed and four miRNAs (*MIR1247-5p*, *MIR204-5p*, *MIR210-3p*, and *MIR375*) were underexpressed in SSA/Ps compared to HPs (Figure 2).

3.2.3 | Control colon comparisons—We compared gene expression in the uninvolved right colon of patients with SSA/Ps ($n = 10$, five of whom had SPS) with control right colon ($n = 15$) from screening colonoscopy patients with no polyps. No difference in gene expression was observed in small RNAs in uninvolved colon from patients with SSA/Ps compared to control colon from patients without polyps. In contrast, when comparing control right ($n = 15$) and left colon ($n = 15$) we identified one miRNA (*MIR615-3p*) differentially expressed more than 20-fold in right colon (Supporting Information Table S2).

3.3 | Sensitivity and specificity analysis of *MIR31-5p*

The sensitivity and specificity of *MIR31-5p* in differentiating SSA/Ps from HPs were determined using a ROC curve. The AUC for the accurate classification of serrated polyps ranged from 76.9% to 100% (Supporting Information Figure S2).

3.4 | qPCR validation of differentially expressed miRNAs

RT-qPCR analysis of five target miRNAs (*MIR31-5p*, *MIR21-3p*, *MIR378A-5p*, *MIR151A-3p*, and *MIR135B-5p*) and one reference gene (*MIRLET7G-5p*) identified four of the five miRNAs differentially expressed in one or more comparisons (Figure 3). *MIR31-5p*, *MIR378A-5p*, and *MIR135B-5p* were differentially expressed in SSA/Ps compared to both uninvolved and control colon. *MIR31-5p* and *MIR135B-5p* were more highly expressed (26.2-fold and 2.4-fold, respectively) in SSA/Ps compared to HPs. Increased expression of *MIR31* and *MIR135B* in SSA/Ps was further confirmed in a separate set of 26 SSA/P and 21 HP FFPE samples, where *MIR31-5p* and *MIR135B-5p* were overexpressed 129.2-fold and 2.5-fold in SSA/Ps compared to HPs, respectively (Supporting Information Figure S3). *MIR31-5p* expression was also higher (8.7-fold) in SSA/Ps compared to APs. *MIR151A-3p* expression was statistically lower in APs compared to uninvolved colon. *MIR21-3p* was not significantly differentially expressed in any comparison although showed the highest expression in the SSA/P group. A full list of fold changes and *P* values for all group comparisons is in Supporting Information Table S3. The correlation plots comparing the

same RNA later-stored polyp samples' small RNA-Seq reads and RT-qPCR fold change for *MIR31-5p* and *MIR135B-5p* are shown in Supporting Information Figure S4. The R^2 values for goodness of fit were 0.976 and 0.786 for *MIR31-5p* and *MIR135B-5p*, respectively.

3.5 | Comparison to TCGA colon cancer RNA-seq data

We investigated the expression of 24 miRNAs, differentially expressed in SSA/Ps (FDR < 0.05, Supporting Information Tables S1 and S2), in 212 colon cancer miRNA datasets from the TCGA. Nine of twenty-four miRNAs (38%) differentially expressed in SSA/Ps were also differentially expressed (Fold = 1.5, FDR < 0.05) in microsatellite-unstable (MSI) cancers compared to microsatellite stable cancers (Supporting Information Table S4). Eight of twenty-four miRNAs (33%) were differentially expressed in CIMP cancers compared to cancers without CIMP (Table 2). Eight and three of twenty-four miRNAs were differentially expressed in *BRAF*-mutant or *KRAS*-mutant cancers compared to cancers without mutations in *BRAF* or *KRAS*, respectively (Table 2). Five SSA/P-specific miRNAs, *MIR9*, *MIR31*, *MIR615*, *MIR584*, and *MIR196B*, were differentially expressed in both CIMP-positive and *BRAF*-mutant colon cancers.

We also investigated the expression of these 24 miRNAs in the recently described CMSs of colon cancer.²⁵ The same 212 TCGA colon cancer datasets were previously classified by CMS subtype (CMS1, $n = 35$; CMS2, $n = 66$; CMS3, $n = 32$; CMS4, 60; not classified, $n = 19$). Sixteen of twenty-four miRNAs differentially expressed in SSA/Ps were also differentially expressed (Fold = 1.5, FDR < 0.05) in one or more of the 4 CMS subtypes. The majority of miRNAs (12/16 miRNAs, 75%) were differentially expressed in CMS1 compared to CMS2 colon cancers (Supporting Information Table S4). Eight of sixteen miRNAs (50%) were differentially expressed in CMS3 cancers, and nine of sixteen miRNAs (56%) were differentially expressed in CMS4 cancers (Supporting Information Table S4). The direction of fold change and overlap of each miRNA according to CMS subtype is shown in Figure 4A. All eight miRNAs differentially expressed in CMS3 cancers were also differentially expressed in CMS1 cancers. In contrast, only four of nine miRNAs differentially expressed in CMS4 cancers were also differentially expressed in CMS1 cancers.

Hierarchical clustering of 24 miRNA genes' expression in TCGA colon cancers by genomic aberration and CMS is shown in Supporting Information Figure S5. Only miRNAs differentially expressed in SSA/Ps were compared. Two distinct miRNA clusters are shown. In CIMP-H, MSI and CMS1 colon cancers, five miRNAs (*MIR9*, *MIR196B*, *MIR204*, *MIR335*, and *MIR1247*) are downregulated and five miRNAs (*MIR31*, *MIR210*, *MIR375*, *MIR584*, and *MIR615*) are upregulated compared to the other cancer subtypes. In contrast, these same 10 miRNAs are expressed in opposing directions in CIMP-negative, microsatellite-stable (MSS), and CMS2 colon cancers. Hierarchical clustering of only these 10 miRNAs is shown in Figure 4B. The heatmap shows that *BRAF* mutation is also associated with the differential expression of these 10 miRNAs and suggests that these miRNAs may be used as a gene panel to identify CMS1 colon cancers with CIMP-H, MSI, and *BRAF* genomic aberrations.

3.6 | Pathway analysis of putative mRNA targets

Using the miRNA target filter application in Ingenuity Pathway Analysis (IPA) software, we compared our miRNA data with previously published messenger RNA data from SSA/Ps.⁷ We identified 1390 mRNA targets with expression that was negatively correlated with one or more miRNAs differentially expressed in SSA/Ps. These 1390 mRNAs were further evaluated for canonical pathway enrichment, and the top 10 pathways are shown in Table 3. Among the top pathways activated were ephrin receptor, insulin growth factor receptor, and ciliary neurotrophic factor signaling. Among the top downregulated pathways were PTEN and PPAR α signaling. Enriched canonical pathways for all 5029 mRNAs differentially expressed in SSA/Ps are shown in Supporting Information Table S5. There was significant overlap in the canonical pathways identified using all differentially expressed mRNAs (5029) and only those targeted by miRNAs (1390) by IPA.

4 | DISCUSSION

SSA/Ps are pre-neoplastic colon polyps with an increased risk for advanced neoplasia and colon cancer. Historically, the neoplastic potential of SSA/Ps has not been appreciated, and SSA/Ps were often grouped with HPs due to significant histological and morphological overlap. Now that HPs (benign) and SSA/Ps (pre-neoplastic) are recognized to harbor different cancer risks, precise diagnostic tools that can inform an individual's cancer risk and drive recommendations on frequency of surveillance are very important. This study is the first to examine small RNA expression using RNA sequencing technology in prospectively collected serrated polyps. Small RNA profiles can be used to distinguish neoplastic from benign lesions.²⁷ We found many differentially expressed miRNAs in SSA/Ps that distinguish them from HPs including *MIR31*, *MIR135B*, *MIR1247*, and *MIR204*. The expression of *MIR31* and *MIR135B* was further validated using RT-qPCR. We also describe for the first time the expression of SSA/P-specific miRNAs in colon cancer CMS²⁵ and the canonical pathways likely targeted by these miRNAs.

The expression of *MIR31*-5p was 26 to 129-fold higher by RT-qPCR in SSA/Ps compared to HPs and 45-fold higher compared to control colon. Higher *MIR31* expression occurs in advanced-stage or aggressive CRCs.^{28–30} *MIR31* expression is associated with decreased progression-free survival (time from initiating chemotherapy until time of death or progression of disease) in metastatic CRC patients treated with anti-EGFR therapy.³¹ *MIR31*-5p promotes proliferation, tumor invasion, and cell survival in the development of CRC.^{29,32,33} *MIR31* has also been detected in serum of patients with CRC³⁴ and is more highly expressed in MSI-H CRCs.³⁵ Recent studies have shown increased *MIR31* expression in SSA/Ps suggesting *MIR31* plays a role in the serrated pathway to colon cancer.^{16,36} A study by Noshro and colleagues¹⁶ showed a positive association of *MIR31* expression with BRAF mutation and poor colon cancer prognosis.

To our knowledge, our study is the first to show overexpression of *MIR135B* in SSA/Ps. *MIR135B* expression is frequently elevated in colon cancer.³⁰ Dysregulation of *MIR135B* has been associated with *APC* dysfunction that may lead to the development of hereditary or sporadic colon cancers.^{37,38} *MIR135B* promotes tumor proliferation, progression, and invasion.³⁸ Increased *MIR135B* levels are found in the stools of patients with colorectal

adenomas when compared to patients with normal colon mucosa.³⁹ In one study, higher *MIR135B* expression in tumor tissue also correlated with improved disease-free and cancer-specific survival (time until tumor related death) in patients with rectal cancer.⁴⁰

Increased *MIR21* expression occurs in CRC and correlates with overall reduced survival and unfavorable prognosis.^{41,42} Schmitz and colleagues⁴³ showed increased expression of *MIR21* in both SSA/Ps and HPs. Evidence suggests it plays a role in cell proliferation and invasion in CRC by targeting the *PTEN* gene.⁴⁴ Our qPCR validation of SSA/P-specific miRNA expression showed that *MIR21* was elevated (1.5-fold) in SSA/Ps but was not statistically significant.

Other miRNAs differentially expressed in SSA/Ps in our study were *MIR584*, *MIR3614-5p*, and *MIR378A-3p*. *MIR584* expression has not been previously reported in serrated polyps or serrated carcinoma. *MIR3614-5p* is highly expressed in cervical tumor samples and distinguishes them from normal tissue.⁴⁵ No data on *MIR3614-5p* expression are available in colon cancer tissue or polyps. Our study is the first to show increased expression of *MIR3614-5p* in SSA/Ps. *MIR378A-3p* was significantly downregulated in SSA/Ps compared to paired uninvolved colon. Similar downregulation of *MIR378* was found in CRC by microarray and RT-qPCR.^{46,47} Decreased expression of *MIR378* was also observed in *KRAS*-mutant colon cancers.⁴⁸

Evaluation of SSA/P-specific miRNA expression in TCGA colon cancer datasets identified many miRNAs that are differentially expressed in CIMP positive colon cancers compared to CIMP negative cancers (Table 2). The expression of *MIR9*, *MIR31*, *MIR196B*, *MIR584*, and *MIR615* is highly correlated with CIMP tumor status and *BRAF* mutation. These changes in expression were most prominent in CIMP high (H) compared to CIMP low (L) colon cancers. However, *MIR9* and *MIR31* show changes in both CIMP-H and CpG island methylator phenotype-low (CIMP-L) colon cancers, suggesting a gradation in expression of these miRNAs according to the level of CpG island methylation. *MIR31* and *MIR584* were also differentially expressed in *KRAS*-mutated cancers, which further supports the idea that *KRAS* mutations are genetic events that contribute to the serrated neoplasia pathway.

MIR9 has been reported to function in CRC as a tumor suppressor by suppressing cell migration and decreasing tumor proliferation.^{32,49} Decreased expression of *MIR9* has been noted in colon adenomas compared to normal colon.⁴⁷ Our small RNA-seq data show similarly downregulated *MIR9* in adenomas (Fold < -1.8, FDR < 0.003). *MIR9* expression has been associated with colon cancer patient survival.⁵⁰ Methylation of the *MIR9* promoter frequently occurs in colon cancer and is associated with lymph node metastasis.⁵¹

The expression of *MIR196B* and *MIR615* is also highly correlated with MSI tumors, suggesting that these miRNAs are associated with colon cancers that develop through the serrated pathway. These findings are in agreement with two previous studies comparing miRNA expression in MSI and stable colon cancers.^{52,53} *MIR196* has been implicated in a variety of gastrointestinal cancers, and its upregulation was noted in CRC.⁵⁴ It has also been detected in plasma of patients with colon cancer.⁵⁴ Our analysis of *MIR196B* suggests that reduced expression is associated with SSA/Ps and CIMP-positive, MSI colon cancers.

Increased expression of *MIR615-3p* has been documented in poorly differentiated and more proximal colon cancer.⁵⁵ It should be noted that *MIR615* is more highly expressed in proximal than distal colon, and therefore differences in *MIR615* expression among colon cancer subtypes may reflect differences in tumor location.

SSA/P-specific miRNA expression also overlapped with one or more colon cancer CMSs, most notably CMS1 (Figure 4A,B). CMS1 colon cancers have been previously described as CIMP-positive, microsatellite-unstable, and *BRAF*-mutated.²⁵ *MIR31*, *MIR196B*, and *MIR615* expression is highly correlated with CMS1 cancers and each of these genomic aberrations. These three miRNAs were also differentially expressed in CMS3 and CMS4 cancers, suggesting that the serrated neoplasia pathway overlaps with other cancer subtypes.

The CMS3 subtype of colon cancer is characterized by a CIMP-L pattern of DNA methylation and frequent mutations in *KRAS*.²⁵ These genomic aberrations have been considered part of an alternative serrated pathway to cancer, although the precursor lesions that harbor these changes are not well defined.⁵⁶ Interestingly, this alternative serrated pathway to cancer may account for the majority of serrated colon cancers and include up to 20% of all colon cancers.⁵⁶ In contrast, only 15% of colon cancers are believed to develop from the classic serrated pathway that includes *BRAF* mutation, CIMP-H DNA methylation, and MSI through silencing of *MLH1*.

MIR31 is overexpressed in CMS1, CMS3, and CMS4 colon cancers compared to CMS2 cancers (Supporting Information Table S4). In fact, all eight miRNAs differentially expressed in CMS3 cancers were also differentially expressed in the same direction in CMS1 colon cancers, suggesting significant molecular overlap between these two cancer subtypes. *MIR31*, *MIR135B*, and *MIR584* were overexpressed in both *BRAF*-mutated and *KRAS*-mutated colon cancers, also suggesting molecular overlap between CMS1 and CMS3 subtypes. *MIR31* showed increased expression in CIMP-L cancers, also consistent with the CMS3 subtype.

CMS4 subtype colon cancers are characterized by activation of transforming growth factor beta (TGFB) signaling and upregulation of genes involved in epithelial-to-mesenchymal transition (EMT).²⁵ These cancers have high levels of somatic copy number alterations, are MSS and have infrequent CIMP DNA methylation.²⁵ The majority of miRNAs (8 of 11, 73%), including *MIR135B*, are underexpressed in CMS4 colon cancers compared to CMS2 cancers. When compared to the mean expression of all other CMS subtypes (CMS1, CMS2, and CMS3), *MIR31* and *MIR584* are also underexpressed in CMS4 cancers (Figure 4B). These data suggest that the miRNA profile in CMS1 colon cancers is more similar to CMS3 than CMS4 cancers.

The canonical signaling pathways affected in SSA/Ps are consistent with enhanced growth factor signaling (Table 3). Ephrin receptor and insulin-like growth factor 1 signaling are increased while PPAR α and PTEN signaling are decreased, each suggesting enhanced phosphoinositide 3 kinase (PI3K) signaling in SSA/Ps. Concomitant *Pten* and *Kras* mutations in mice increase cell proliferation along the crypt-villus axis, branching of intestinal villi and the formation of HPs and sessile serrated adenomas.⁵⁷ Sessile serrated

adenomas from these mice also show increased levels of phosphorylated AKT, ERK, and MEK protein, suggesting increased PI3K signaling.⁵⁷

Colon cancers with *PI3KCA* exon 20 or *PTEN* mutations are associated with a proximal location, *BRAF* mutation, MSI and are CIMP-H,⁵⁸ suggesting that these cancers develop through the sessile serrated pathway. Similarly, *PIK3CA* exon 9 mutations are also common in proximal colon cancers but are associated with a CIMP-L phenotype and *KRAS* mutation,⁵⁸ suggesting an alternative serrated pathway to colon cancer. These findings are consistent with the miRNA profiles and signaling pathways we have found altered in SSA/Ps. However, mutations in *PIK3CA* and *PTEN* have not been described in SSA/Ps, which warrants further research in this area.

TGFB signaling, also increased in SSA/Ps, primarily induces EMT in *BRAF*-mutated colon organoids but not organoids derived from traditional adenomas.⁵⁹ It has been hypothesized that CMS4 subtype colon cancers may develop from SSA/Ps due to common gene signatures in TGFB signaling, matrix remodeling, and EMT pathways.⁶⁰ NANOG expression is influenced by both TGFB and PI3K signaling,^{61,62} and the observed increase in NANOG signaling in SSA/Ps likely contributes to EMT. Overexpression of NANOG has been associated with advanced tumor stages and poor survival in colon cancer patients.⁶³

In summary, this report provides a comprehensive analysis of miRNA gene expression in prospectively collected serrated colon polyps. We identified previously undescribed, differential expression of miRNAs in SSA/Ps but not HPs, including *MIR135B*, *MIR378A*, *MIR548*, *MIR9*, and *MIR196B*, suggesting that miRNAs are good predictors of serrated neoplasia. Interestingly, the majority of SSA/P-specific miRNAs are also differentially expressed in CMS1-subtype colon cancers that are CIMP-positive, *BRAF*-mutated, and MSI. *MIR9*, *MIR31*, and *MIR196B* are also differentially expressed in CMS3-subtype colon cancers, suggesting that the serrated pathway may encompass multiple colon cancer subtypes. Pathway analysis of miRNA targets suggests that activation of growth factor signaling pathways underlie serrated polyp biology. Future mechanistic and clinical studies will be required to study the significance of these miRNA profiles in colon cancer developing from SSA/Ps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Relative expression of 23 miRNAs differentially expressed in SSA/Ps across 26 serrated polyps (16 SSA/Ps and 10 HPs). A, Hierarchical clustering was performed with correlation and single linkage metrics using log₂ ratios for each polyp compared to matched uninvolved colon. Four distinct miRNA gene clusters were identified. B, Principal component analysis (PCA) of the 26 polyp samples using the same log₂ ratio values as in A. Principal component 1 (PC1), PC2, and PC3 accounted for 19%, 12%, and 10% of the variation observed in the gene expression data, respectively. SSA/P red and HP blue filled circles [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 2.

Relative expression of 6 miRNAs differentially expressed in 16 SSA/Ps compared to 14 HPs. Four miRNAs were underexpressed (filled circles, fold change between -1.77 and -4.40) and two were overexpressed (filled triangles, fold change between 7.11 and 11.16) in SSA/Ps compared to HPs. Average read counts for each serrated polyp type are shown on a log scale (SSA/P - Y axis, HP - X axis)

FIGURE 3.

Expression of *MIR31-5p*, *MIR21-3p*, *MIR378A-5p*, *MIR151A-3p*, and *MIR135B-5p* by reverse transcription quantitative PCR analysis. Expression of each miRNA was normalized to *MIRLET7G-5p* expression and fold change (y axis) determined relative to the mean of control colon values. Individual sample values and the mean $\pm$ SE for each of seven sample groups (x axis) are shown. “AP” denotes adenomatous polyp and “unin” denotes uninvolved colon. The number of samples analyzed in each group is shown in parentheses. Statistical significance was calculated using the Mann-Whitney *U*-test. ns denotes not significant

FIGURE 4.

SSA/P-specific miRNA expression in TCGA colon cancers according to genomic aberration and consensus molecular subtype (CMS). A, Venn diagram showing the overlap in 16 miRNAs differentially expressed (Fold ≥ 1.5 , FDR < 0.05) according to CMS subtype. Direction and significance of fold change expression for each miRNA were calculated by comparing CMS1, CMS3, and CMS4 subtypes to the CMS2 (conventional adenocarcinoma) subtype. MiRNAs in white and red text show the same and opposite direction of fold change in SSA/Ps compared to paired uninvolved colon, respectively. B, Hierarchical clustering of SSA/P-specific miRNA expression in 212 TCGA colon cancers according to genomic aberration and CMS subtype. Two distinct colon cancer subtype clusters (horizontal identifiers) and two miRNA gene clusters (vertical identifiers) were identified. Heatmap represents the log₂ ratio of the mean for colon cancers with a specific genomic aberration or CMS subtype compared to the mean of all other cancers without that genomic aberration or all other CMS subtypes, respectively [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

miRNAs differentially expressed in SSA/Ps, APs, and/or HPs compared to paired uninvolved colon

miRNA	SSA/P_Fold	SSA/P_FDR	AP_Fold	AP_FDR	HP_Fold	HP_FDR
MIR31-5p	9.14	<0.001	2.82	0.157	1.98	0.527
MIR135B-5p	5.42	<0.001	6.57	0.001	1.98	0.407
MIR549A	4.18	0.012	15.56	<0.001	1.00	1.000
MIR3614-5p	2.63	0.006	-1.32	0.443	1.78	0.407
MIR222-5p	2.37	0.018	-1.23	0.803	1.48	0.670
MIR144-3p	2.30	<0.001	1.66	0.409	-2.04	0.407
MIR584-5p	2.18	0.004	2.07	0.038	1.67	0.407
MIR451A	2.10	0.014	1.32	0.637	-1.16	0.892
MIR4488	2.09	0.005	1.07	0.916	2.25	0.010
MIR151A-5p	-2.24	0.015	1.02	0.939	-1.12	0.870
MIR205-5p	-3.93	0.011	1.00	1.000	1.00	1.000

Eleven, three, and one miRNA(s) was (were) differentially expressed more than twofold in SSA/Ps ($n = 16$), APs ($n = 14$), and HPs ($n = 10$), respectively. Values for each differentially expressed miRNA are shown across all three polyp subtypes. FDR denotes Benjamini and Hochberg false discovery rate.

TABLE 2
SSA/P-specific miRNAs differentially expressed in CIMP positive, BRAF, and KRAS mutant colon cancers

MIRNA	CIMPH (n = 37) Fold	CIMPL (n = 47) Fold	CIMP FDR	BRAF (n = 31) Fold	BRAF FDR	KRAS (n = 86) Fold	KRAS FDR
MIR9	-3.205	-1.859	<0.001	-2.000	<0.001	-1.214	0.583
MIR210	2.308	1.321	<0.001	1.608	0.082	1.004	0.993
MIR31	3.286	2.181	<0.001	2.125	0.019	2.593	<0.001
MIR615	3.748	-1.042	<0.001	6.559	<0.001	1.503	0.401
MIR584	1.916	1.193	0.002	2.249	<0.001	1.673	<0.001
MIR375	1.790	1.474	0.004	1.209	0.520	1.079	0.834
MIR196b	-1.721	-1.424	0.005	-2.907	<0.001	-1.375	0.235
MIR135b	1.654	1.097	0.030	1.583	0.051	1.769	<0.001
MIR335	-1.213	1.076	0.055	-1.727	<0.001	-1.137	0.463
MIR1247	-1.101	1.477	0.541	-2.955	<0.001	-1.312	0.423
MIR204	-1.030	-2.232	0.574	-2.358	0.023	1.363	0.503

Eight miRNAs showed a significant linear change in expression (Fold 1.5, FDR < 0.05) in CIMP high (H) and CIMP low (L) cancers by analysis of trend. Fold change values represent the mean expression of each miRNA in 37 CIMP-H and 47 CIMP-L cancers compared to 128 CIMP-negative cancers. Eight and three miRNAs showed a significant change (DESeq2 statistics) in BRAF (n = 31) and KRAS (n = 86) mutant cancers compared to cancers without BRAF or KRAS mutations (n = 91), respectively. Four cancers had both BRAF and KRAS mutations and were excluded from BRAF and KRAS statistical analyses.

TABLE 3
Ingenuity pathway analysis of 1390 mRNA targets differentially expressed in SSA/Ps compared to paired uninvolved colon

Canonical pathways	Total genes	SSA/P (<i>n</i> = 9, 1390 genes)	
		Gene overlap (%)	<i>P</i> value
Ephrin receptor signaling	175	28 (16)	3.17E-06
Insulin growth factor 1 signaling	106	19 (18)	2.33E-05
Oncostatin M signaling	34	9 (26)	1.60E-04
Ciliary neurotrophic factor signaling	63	12 (19)	4.05E-04
Interleukin 7 signaling	91	14 (15)	1.31E-03
NANOG stem cell signaling	122	17 (14)	1.32E-03
ERK5 signaling	66	11 (17)	2.18E-03
Transforming growth factor beta signaling	87	13 (15)	2.51E-03
PPAR alpha/retinoic X receptor alpha	180	21 (12)	3.67E-03
PTEN signaling	119	14 (12)	1.48E-02

Each of the 1390 mRNAs are targets of one or more SSA/P-specific miRNAs as determined by HumanTargetScan. *P*-values represent the hypergeometric test of enrichment based on the number of genes differentially expressed in the pathway compared to the total number of genes in the pathway. *z* score represents whether the pathway is activated (>2.0) or inhibited (<-2.0) based on the direction of fold change for each differentially expressed gene in that pathway.