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MicroRNAs Associated With Biological Pathways of Left- and Right-sided Colorectal Cancer

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Abstract. *Background/Aim: MicroRNAs (miRNAs) regulate the development of colorectal cancer (CRC). We aimed to investigate miRNAs and their relation to cancer-related signaling pathways in site-specific CRC. Materials and Methods: We used a total of 24 left- and right-sided Finnish CRC samples (discovery cohort) and The Cancer Genome Atlas public mature miRSeq dataset of 201 CRC samples (validation cohort). MiRNA differential expression and biological pathway analyses were performed using DESeq2 and the DIANA/mirPath tool, respectively. Results: We found 17 significantly differentially up-regulated [false discovery rate (FDR) <0.05] miRNAs in left-sided CRC ("left miRNAs"), and 15 in right-sided CRC ("right miRNAs"). The left miRNAs participate in the mTor, Wnt, PI3K-Akt signaling pathways (FDR<0.05). The right miRNAs participate in the TGF-β signaling pathway. We also observed that both cohorts share six miRNAs. One of these (hsa-miR-196b-5p) was significantly (FDR<0.05) up-regulated in left-sided CRC. The rest of them (hsa-miR-625-3p, hsa-miR-155-5p, hsa-miR-625-5p, hsa-miR-31-5p and hsa-miR-330-5p) showed significant (FDR<0.05) up-regulation in right-sided CRC. Conclusion: Left and right miRNAs are associated with predominant biological pathways of left- and right-sided CRC, respectively. Our results may be beneficial for classifying CRC and for future biomarker studies of site-specific CRC.*

Colorectal cancer (CRC) can be divided into left- and right-sided CRC. The right-sided tumors form in the cecum, ascending colon or in the two thirds of the transverse colon, whereas the left-sided tumors develop in the distal third of the transverse colon, descending colon, sigmoid colon or rectum (1, 2). This classification solely by the anatomic location is a simplification. Biological heterogeneity of CRC also has a role in its classification. Classification by the embryonic development, where the right and left colon develop from the midgut and hindgut, respectively, lead to differences in biological pathways of left- and right-sided CRC (1). In fact, certain biological pathways are predominant in either left- or right-sided CRC (Table S1). For example, *TP53* mutation (3) and the phosphatidylinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K-Akt-mTor) (4, 5) signaling pathway are predominant in left-sided CRC. In contrast, serine/threonine kinase (*BRAF*) mutation (6) and microsatellite instability (MSI) (7) are prevalent in right-sided CRC. Differences in predominant biological pathways may also classify CRC into two subgroups (7).

MicroRNAs (miRNAs) have been suggested to be potential molecular classifiers and biomarkers in CRC. MiRNAs are small non-coding RNAs involved in

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posttranscriptional regulation. They participate in critical biological processes in CRC including cell growth, proliferation and metastasis (8), and some of their expression levels in CRC are up- or down-regulated compared to nontumor tissues (9). For example, many studies have reported that the expression levels of miR-21, miR-31 and miR-20a are up-regulated in CRC, whereas the expression of miR-143 and miR-145 are down-regulated (9). Additionally, changes in miRNA expression patterns can classify different tumor types, such as breast, lung and colon cancers (10). Therefore, miRNA profiles may help in classifying left- and right-sided CRC and may benefit studies of non-invasive biomarkers aimed at detecting site-specific CRC.

In this study, we aimed to elucidate the biological characteristics of left and right-sided CRC by studying their miRNA expression levels. We assumed that different miRNA expression levels influence the biological pathways of the two forms of CRC. Our results showed different miRNA profiles in the two forms of CRC and their participation in predominant biological pathways of site-specific CRC. This may bring new insights into the classification of CRC and, thus, may help to develop site-specific miRNA biomarkers.

Materials and Methods

Discovery cohort. A total of 24 CRC tumor samples were collected during primary cancer surgeries at the Central Finland Central Hospital in Jyväskylä, Finland. The samples are from Finnish CRC patients and a surgical pathologist selected the samples. The right-sided CRC samples (N=12) were obtained from the cecum and ascending colon, and the left-sided CRC (N=12) samples from the sigmoid colon and rectum. The clinicopathologic features of the samples in this discovery cohort are shown in Table I. The collection of the CRC samples and their research use was approved by the Central Finland Health Care District (Jyväskylä, Finland). All patient data were pseudonymised before access to researchers was granted.

Validation cohort. We used 201 samples of The Cancer Genome Atlas (TCGA) mature miRSeq dataset (TCGA data version 2016_01_28 for COADREAD) provided by FireBrowse (11) for validation purposes (Table I). From TCGA samples, we selected the cecum and ascending colon for the right-sided CRC samples and the sigmoid colon and rectum for the left-sided CRC samples.

Microsatellite instability analysis. We performed immunohistochemical expression analysis of the four mismatch repair (MMR) proteins [MutL homolog 1 (MLH1), postmeiotic segregation increased 2 (PMS2), MutL homolog 2 (MSH2), and MutL homolog 6 (MSH6)] to determine MMR status as previously described (12). The following commercially available antibodies were used: MLH1 (Novocastra NCL-L-MLH1, clone ES05), MSH2 (Oncogene Research Products NA27, clone FE11), MSH6 (Cell Marque 287M-16, clone 44), PMS2 (BD Pharmingen 556415, clone A16-4). The loss of expression of a single protein or heterodimer couple suggests the presence of MMR deficiency. MMR deficient cases were classified as cases with microsatellite instability (MSI). Tissue samples that showed positive staining for all four markers were considered microsatellite stable (MSS) (13).

RNA isolation. We extracted total RNA containing miRNA using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) from the fresh frozen CRC tissues (~10 to 45 mg) of the discovery cohort. We used a NanoDrop ND-1000 UV/Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) with the Qubit RNA BR (Broad-Range) Assay Kit (Thermo Fisher Scientific) for the RNA concentration measurement. The quality of total RNA was analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with the Agilent RNA 6000 Nano Kit (Agilent Technologies). The average of RIN values of the samples used for a library preparation was 7.7 (range=5.3-9.6). RIN value of the samples was higher than 7 for 87.5% of the samples.

Library preparation and sequencing. We constructed small RNA libraries using the TruSeq® Small RNA Library Prep Kit (Illumina, San Diego, CA, USA). The quality of the twenty-four generated DNA libraries was analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies) using the Agilent High Sensitivity DNA Kit (Agilent Technologies). We pooled twelve DNA libraries together in an equimolar manner resulting in two pools from the 24 samples. The pools were sequenced using the MiSeq System (Illumina) with the MiSeq Reagent Kit v3 150 cycles (Illumina).

Sequencing data analysis. We used an in-house bioinformatic analysis pipeline (Figure S1) to analyze the sequencing data of the discovery cohort. The first steps of the pipeline included read quality assessment (FastQC, v.1.0.1), adapter trimming (Trimmomatic, v.1.0.1) and removal of ribosomal RNA reads (Bowtie2, v.2.2.3). The remaining reads were then aligned (Tophat2 v.2.0.13) to a human mature miRNA (hg38) from miRbase (v.21) (14) followed by data conversions for data visualization (IGVtools, v.2.3.32 and IGV, v.2.3.49). After alignment, quantitation (GenomicAlignments, v1.6.3) and annotation (GenomicRanges, v1.22.4) were performed using R (v.3.2.2). The initial, unprocessed read counts ranged from 773 727 to 1 688 647 per sample, out of which 655 156 to 1 294 352 reads survived preprocessing, and 624 249 to 1 227 474 was aligned. All samples had high mean Sanger 1.9 encoded sequence quality scores, ranging from 33.59 to 36.05 for the initial, unprocessed reads and from 36.65 to 37.00 for fully preprocessed reads. Statistical analysis of differentially expressed (DE) miRNAs was carried out to find candidate miRNAs using DESeq2 (v.1.12.4)/RStudio (v.0.99.486) with the Wald statistical test. *p*-Values from the Wald test were corrected for multiple testing using the Benjamini and Hochberg correction (15) that provides a false discovery rate (FDR). MiRNAs with FDR<0.05 were considered statistically significantly DE.

Definition of left and right miRNAs. We categorized candidate DE miRNAs either as left or right miRNAs in both discovery and validation cohorts. If an miRNA had higher expression level in the right-sided CRC samples than in the left-sided ones, with FDR<0.05, it was named as "right miRNA", and likewise for "left miRNA". In other words, "right miRNA" is up-regulated in the right-sided CRC samples and down-regulated in the left-sided CRC samples, and "left miRNA" is up-regulated in left-sided CRC and down-regulated in right-sided CRC. Because the main purpose of this study was to compare the differences in miRNA expression levels between the left- and right-sided CRC samples, we set right-sided CRC samples as a reference group in the DESeq2 analysis for

Table I. *Clinicopathologic parameters of colorectal cancer patients*.

	Discovery cohort			Validation cohort		
Clinical parameters	Total N (%)	Left N (%)	Right N (%)	Total N (%)	Left N (%)	Right N (%)
Number of tumor samples	24 (100%)	12 (50%)	12 (50%)	201 (100%)	100 (50%)	101 (50%)
Age						
<70	12 (50%)	6 (50%)	6 (50%)	114 (57%)	62 (54%)	52 (46%)
≥70	12 (50%)	6 (50%)	6 (50%)	87 (43%)	38 (44%)	49 (56%)
Gender						
Female	9 (37.5%)	4 (44%)	5 (56%)	89 (44%)	47 (53%)	42 (47%)
Male	15 (62.5%)	8 (53%)	7 (47%)	112 (56%)	53 (47%)	59 (53%)
Tumor location						
Sigmoid/Rectum	12 (50%)	5(42%)/7(58%)	-	100 (50%)	67 (67%)/33 (33%)	-
Ascending/Cecum	12 (50%)	-	4 (33%)/8 (67%)	101 (50%)	-	43 (43%)/58 (57%)
Ethnicity						
White	24 (100%)	12 (50%)	12 (50%)	157 (78%)	79 (50%)	78 (50%)
American Indian or Alaska native	-	-	-	1 (0.5%)	1 (100%)	0 (0%)
Asian	-	-	-	9 (4.5%)	3 (33%)	6 (67%)
Black or african american	-	-	-	17 (8.5%)	3 (18%)	14 (82%)
NA	-	-	-	17 (8.5%)	14 (82%)	3 (18%)
Depth of invasion						
Tis	-	-	-	1 (0.5%)	0 (0%)	1 (100%)
T1	-	-	-	7 (3.5%)	4 (57%)	3 (43%)
T2	5 (21%)	4 (80%)	1 (20%)	31 (15.4%)	14 (45%)	17 (55%)
T3	17 (71%)	6 (35%)	11 (65%)	139 (69.2%)	69 (50%)	70 (50%)
T4-T4b	2 (8%)	2 (100%)	0 (0%)	23 (11.4%)	13 (57%)	10 (43%)
Regional lymph node status						
NX	-	-	-	1 (0.5%)	1 (100%)	-
N0	16 (66.7%)	9 (56%)	7 (44%)	113 (56.2%)	53 (47%)	60 (53%)
N1-N1c	3 (12.5%)	0 (0%)	3 (100%)	52 (25.9%)	33 (63%)	19 (37%)
N2-N2b	5 (20.8%)	3 (60%)	2 (40%)	35 (17.4%)	13 (37%)	22 (63%)
Stage						
Stage I-IA	5 (21%)	4 (80%)	1 (20%)	32 (15.9%)	14 (44%)	18 (56%)
Stage II-IIC	11 (46%)	5 (45%)	6 (55%)	76 (37.8%)	38 (50%)	38 (50%)
Stage III-IIIC	8 (33%)	3 (38%)	5 (63%)	57 (28.4%)	29 (51%)	28 (49%)
Stage IV-IVB	-	-	-	27 (13.4%)	16 (59%)	11 (41%)
NA	-	-	-	9 (4.5%)	3 (33%)	6 (67%)
Microsatellite instability						
MSI	4[a] (17%)	0 (0%)	4[a] (100%)	61[b] (30%)	20[b] (33%)	41[c] (67%)
MSS	19 (79%)	12 (63%)	7 (37%)	140 (70%)	80 (57%)	60 (43%)
NA	1 (4%)	-	1 (100%)	-	-	-

[a]MutL homolog 1 (MLH1) and post meiotic segregation increased 2 (PMS2) negative; [b]Mononucleotide and dinucleotide marker analysis; [c]The 41 MSI cases can be further divided into 27 MSI-H cases and into 14 MSI-L cases. NA: Not available; MSI: microsatellite instability; MSI-H: microsatellite instability-high; MSI-L: microsatellite instability-low; MSS: microsatellite stable.

differential gene expression. Therefore, negative log2 transformed fold change (FC) values represent right miRNAs and positive log2 transformed FC values left miRNAs.

Pathway enrichment analysis. We used the DIANA/mirPath (v.3) tool (16) to study the biological pathways of the selected (FDR<0.05) left and right miRNAs of the discovery and validation cohorts. The DIANA mirPath tool first identifies miRNA target genes and then predicts biological pathways based on target genes. The default settings were used in DIANA/mirPath, but for the database settings, TarBase (v.7.0) was selected. The DIANA/mirPath tool provides adjusted (Benjamini and Hochberg) (15) *p*-values (FDR) for the pathway association. Biological pathways with FDR<0.05 were considered as statistically significant.

Results

Microsatellite instability in right-sided CRC samples. Most of the MSI cases were right-sided CRC both in the discovery and validation cohorts (Table I). There were four MSI cases in the discovery cohort and all were in right-sided CRC. In the validation cohort, 41 (67%) out of the 61 MSI cases were in right-sided CRC and the rest of them (33%) in left-sided CRC.

Table II. *Differentially up-regulated left and right miRNAs in the discovery cohort.*

Left miRNA[a]	Accession number[b]	FC[c]	FDR[d]	Right miRNA[a]	Accession number[b]	FC[c]	FDR[d]
hsa-let-7c-5p	MIMAT0000064	2.53	1.47×10^{-5}	hsa-miR-625-3p	MIMAT0004808	–1.3	3.94×10^{-4}
hsa-miR-99a-5p	MIMAT0000097	2.55	1.47×10^{-5}	hsa-miR-155-5p	MIMAT0000646	–1.21	0.002
hsa-miR-125b-2-3p	MIMAT0004603	2.71	1.67×10^{-5}	hsa-miR-615-3p	MIMAT0003283	–2.78	0.004
hsa-miR-23a-3p	MIMAT0000078	0.57	0.012	hsa-miR-625-5p	MIMAT0003294	–1.13	0.006
hsa-miR-125b-5p	MIMAT0000423	1.65	0.015	hsa-miR-150-5p	MIMAT0000451	–1.76	0.006
hsa-miR-193b-3p	MIMAT0002819	1.17	0.015	hsa-miR-589-5p	MIMAT0004799	–0.75	0.021
hsa-miR-125b-1-3p	MIMAT0004592	1.48	0.018	hsa-miR-31-5p	MIMAT0000089	–1.7	0.025
hsa-miR-145-3p	MIMAT0004601	1.44	0.018	hsa-miR-330-5p	MIMAT0004693	–1.03	0.025
hsa-miR-196b-5p	MIMAT0001080	1.82	0.018	hsa-miR-150-3p	MIMAT0004610	–1.88	0.028
hsa-miR-199b-5p	MIMAT0000263	1.02	0.019	hsa-miR-30e-5p	MIMAT0000692	–0.33	0.028
hsa-miR-218-5p	MIMAT0000275	1.35	0.019	hsa-miR-942-5p	MIMAT0004985	–1.4	0.028
hsa-miR-199a-3p	MIMAT0000232	0.88	0.028	hsa-miR-15a-5p	MIMAT0000068	–0.54	0.034
hsa-miR-199b-3p	MIMAT0004563	0.88	0.028	hsa-miR-345-5p	MIMAT0000772	–0.76	0.039
hsa-miR-9-5p	MIMAT0000441	1.64	0.028	hsa-miR-641	MIMAT0003311	–1.53	0.039
hsa-miR-99a-3p	MIMAT0004511	2.16	0.032	hsa-miR-130b-5p	MIMAT0004680	–0.79	0.049
hsa-miR-218-1-3p	MIMAT0004565	1.86	0.045				
hsa-miR-3074-5p	MIMAT0019208	0.55	0.045				

[a]Left miRNA represents miRNA, which was differentially up-regulated in the left-sided CRC samples and down-regulated in the right-sided CRC samples; Likewise, right miRNA was differentially up-regulated in the right-sided CRC samples and down-regulated in the left-sided CRC samples. [b]miRBase accession number (14); [c]Right-sided CRC samples were set as a reference group in the DESeq2 analysis for differential gene expression. Therefore, negative log2 transformed FC values represent right miRNAs and positive log2 transformed FC values left miRNAs; [d]FDR corrected *p*-value (Benjamini and Hochberg) from the DESeq2 analysis for differential gene expression (Wald test). FC: Fold change (log2 transformed); FDR: false discovery rate; miRNA: microRNA.

Differentially up-regulated left and right miRNAs in the discovery cohort. Our data indicate differences in miRNA expression levels between left- and right-sided CRC (Figure S2). We analyzed the miRNA expression levels between the 12 left-sided and 12 right-sided CRC samples using the DESeq2 statistical analysis. We found 17 left and 15 right differentially up-regulated miRNAs with FDR<0.05 (Table II, Table S2). The most significant left miRNAs (FDR<2×10^{-5}) were hsa-let-7c-5p, hsa-miR-99a-5p, and hsa-miR-125b-2-3p. In the right miRNAs, the most significant miRNA was hsa-miR-625-3p (FDR=3.94×10^{-4}).

Predominant biological pathways of CRC among the left and right miRNAs in the discovery cohort. We used the pathway enrichment analysis to study whether the 17 left and 15 right miRNAs were involved in certain previously published (Table S1) site-specific predominant biological pathways of CRC (Table III) [the top 25 biological pathways of the left miRNAs and of the right miRNAs are shown in Tables S3 and S4, respectively]. We found that the participation of the left miRNAs in the mTor, Wnt, and PI3K-Akt signaling pathways were significant (FDR<0.05) compared to the right miRNAs (Table III). The right miRNAs were involved in the TGF-β signaling pathway more significantly (FRD=6.24×10^{-6}) than the left ones (FRD=0.007). MiRNAs of both CRC sites were involved in the p53 signaling pathway significantly (FDR<0.05), whereas the relation of the left and right miRNAs to the MAPK signaling pathway was not significant.

Predominant biological pathways of CRC among the left and right miRNAs in the validation cohort. We performed the pathway enrichment analysis to see whether the findings in the discovery cohort could be confirmed in the validation cohort (Table III). The validation cohort had 35 left and 16 right differentially up-regulated miRNAs (Table S5). The participation of the left miRNAs in the Wnt signaling pathway was significant (FDR<0.05) compared to the right miRNAs, as found also in the discovery cohort (Table III). The relations of the left and right miRNAs to the p53, mTor, TGF-beta and MAPK signaling pathways were significant (FDR<0.05). The PI3K-Akt signaling pathway was not significantly associated with either miRNA group.

The top (FDR<1×10^{-5}) four biological pathways identified in the pathway enrichment analysis (adherens junction, proteoglycans in cancer, lysine degradation and protein processing in endoplasmic reticulum) using both the left and the right miRNAs were the same in both cohorts (Tables S3, S4, S6 and S7).

Shared miRNAs in the discovery and validation cohorts. The discovery and validation cohorts share six statistically significant (FDR<0.05) miRNAs (Table IV). Five out of the six miRNAs (hsa-miR-625-3p, hsa-miR-155-5p, hsa-miR-625-5p, hsa-miR-31-5p and hsa-miR-330-5p) were differentially up-regulated in right-sided CRC, and one (hsa-miR-196b-5p) in left-sided CRC.

Table III. *Predominant biological pathways of CRC among the left and right miRNAs in the discovery and validation cohorts.*

KEGG pathway	Discovery cohort: Left miRNAs[a] FDR[c]	Discovery cohort: Right miRNAs[a] FDR[c]	Validation cohort: Left miRNAs[b] FDR[c]	Validation cohort: Right miRNAs[b] FDR[c]	Pathway	Predominance in: Left-sided CRC	Predominance in: Right-sided CRC	References
p53 signaling pathway	1.50×10^{-4}	1.01×10^{-5}	5.21×10^{-4}	4.83×10^{-6}	*TP53* mutation	X		(3)
mTOR signaling pathway	0.002	0.388	0.001	0.028	mTOR signaling pathway	X		(4, 5)
Wnt signaling pathway	0.021	0.155	0.002	0.209	Wnt signaling pathway	X		(7)
PI3K-Akt signaling pathway	0.031	0.126	0.083	0.313	PI3K-akt signaling pathway	X		(5)
TGF-beta signaling pathway	0.007	6.24×10^{-6}	6.15×10^{-10}	0.004	TGF-beta signaling pathway with MSI		X	(21)
MAPK signaling pathway	0.506	0.180	0.005	0.002	MAPK signaling pathway		X	(7)

[a]Left and right miRNAs of the discovery cohort are listed in Table II; [b]Left and right miRNAs of the validation cohort are listed in Table S5; [c]FDR corrected *p*-value (Benjamini and Hochberg) from the DIANA/mirPath analysis. Akt: Protein kinase B; CRC: colorectal cancer; FDR: false discovery rate; KEGG: Kyoto Encyclopaedia of Genes and Genomes; MAPK: mitogen-activated protein kinase; miRNA: microRNA; mTOR: mechanistic target of rapamycin; PI3K: phosphatidylinositide 3-kinases; TGF: transforming growth factor.

Table IV. *Shared miRNAs from the validation and discovery cohorts and their association with colorectal cancer pathway.*

miRNA	DE analysis: Discovery cohort FC[a]	Discovery cohort FDR[b]	Discovery cohort Location	Validation cohort FC[a]	Validation cohort FDR[b]	Validation cohort Location	CRC pathway FDR[c,d]
hsa-miR-196b-5p	1.82	0.018	Left	0.8	0.004	Left	0.008
hsa-miR-625-3p	–1.3	3.94×10-4	Right	–0.68	0.003	Right	0.036
hsa-miR-155-5p	–1.21	0.002	Right	–0.43	0.038	Right	0.001
hsa-miR-625-5p	–1.13	0.006	Right	–0.67	0.004	Right	0.995
hsa-miR-31-5p	–1.7	0.025	Right	–0.67	0.007	Right	0.086
hsa-miR-330-5p	–1.03	0.025	Right	–0.52	0.004	Right	0.217

[a]Right-sided CRC samples were set as a reference group in the DESeq2 analysis. Therefore, negative log2 transformed FC values represent right miRNAs, and positive FC values for left miRNAs; [b]FDR corrected *p*-value (Benjamini and Hochberg) from the DESeq2 analysis for differential gene expression (Wald test); [c]FDR corrected *p*-value (Benjamini and Hochberg) from the DIANA/mirPath analysis; [d]Individual miRNA association with CRC. CRC: Colorectal cancer; DE analysis: differential expression analysis; FC: fold change (log2 transformed); FDR: false discovery rate; miRNA: microRNA.

The CRC pathway was one of the significant findings from the pathway enrichment analysis (bolded in Tables S3, S4, S6 and S7). The left hsa-miR-196b-5p was statistically significantly (FDR=0.008) associated with the CRC pathway (Table IV). The right hsa-miR-625-3p and hsa-miR-155-5p were also statistically significant in the CRC pathway with FDR values 0.036 and 0.001, respectively.

Discussion

In addition to the anatomical locations of left- and right-sided CRC, differences also exist in their biological pathways (1, 2, 7, 17). These pathways can be examined using miRNAs, as they are involved in critical biological processes in CRC (8). Our data indicated clear differences in the expression levels of miRNAs between left- and right-sided CRC (Figure S2) that influence predominant biological pathways involved in both forms of CRC.

In the discovery cohort, we identified 17 left and 15 right differentially up-regulated miRNAs (Table II). Our aim was to specifically and directly emphasize the differences between left- and right-sided CRC using miRNAs. For this, we used only malignant tumor tissues. By comparing the miRNA level directly between left and right-sided CRC tumors, we could omit the probable expression variation due to the individual expression patterns of normal tissues. We also validated side-specific miRNA expression in CRC using previously reported scientific results, where normal tissues

were used as controls when calculating the miRNA levels of CRC samples (9). It has been previously shown that the expression levels of let-7, miR-99, miR-125, miR-193, miR-145, miR-196, miR-9, miR-155, miR-150, miR-31, miR-330, miR-30, miR-15 and miR-130 are altered in CRC compared to non-tumor samples, but it has been unknown whether the alterations are present in all of the colon or at more localized regions (9). In our study, we indicated that let-7, miR-125, miR-193, miR-145, miR-196, miR-9 and miR-99 are "left miRNAs" meaning that they are up-regulated in left-sided CRC compared to right-sided CRC. In addition, we categorized miR-155, miR-150, miR-31, miR-330, miR-30, miR-15 and miR-130 as "right miRNAs", indicating their up-regulation in right-sided CRC compared to left-sided CRC. Even though we did not include normal tissues of the colon in our study, we understand that normal tissues could elucidate the miRNA differences between two sides of CRC. For example, Mjelle *et al*. found that miR-615-3p (our right miRNA) was up-regulated in right-sided colon cancer compared to left-sided tumors, however, they also found that this miRNA is up-regulated in right normal colon compared to left normal colon (18). Thus, the expression difference of miR-615-3p between right- and left-sided tumors is most probably due to biological differences between left and right normal colon (18). They also found that miR-196b (our left miRNA) was up-regulated in the right normal colon compared to the left one, but when comparing left and right tumor colons, miR-196 was up-regulated in the left tumor colon, indicating that miR-196b expression is depended on location and the phase of the cell at tumorigenesis (18).

Our site-specific miRNAs are consistent with other researchers' findings. Yang *et al*. found 54 (FDR<0.01) DE miRNAs (22 down-regulated and 32 up-regulated miRNAs) between right-sided colon adenocarcinoma (RSCOAD) and left-sided colon adenocarcinoma (LSCOAD) samples obtained from TCGA (19). We could identify 81% of right miRNAs and 22% of left miRNAs of the validation cohort in their results. The reason, why only 22% of left miRNAs are consistent with the LSCOAD results might be due to the fact that we included the rectum part in our left-sided CRC samples while RSCOAD included only colon adenocarcinomas. Furthermore, they have validated that hsa-miR-224-5p (our left specific miRNA that is up-regulated in left-sided CRC in the validation cohort) is up-regulated in LSCOAD and hsa-miR-155-5p (our right specific miRNA that is up-regulated in right-sided CRC in both cohorts) is up-regulated in RSCOAD using qPCR. Validating DE miRNAs using qPCR confirms the significance of site-specific miRNAs. Our site-specific miRNA findings are also consistent with Hu's *et al*. results (20). They found that eight miRNAs were down-regulated in right colon cancer (RCC), whereas seven miRNAs were up-regulated compared to left colon cancer (LCC). Their four down-regulated miRNAs (miR-196b, miR-552, miR-592 and miR-1247) in RCC were our "left miRNAs", indicating their up-regulation in left-sided CRC and down-regulation in right-sided CRC. In addition, we categorized their five (miR-10b, miR-31, miR-155, miR-615 and miR-625) up-regulated miRNAs in RCC as "right miRNAs" in our results because these miRNAs were up-regulated in the right-sided CRC samples and down-regulated in the left-sided CRC samples. Thus, our study revealed new site-specific miRNAs in site-specific CRC, in accordance with the results of others.

Our left and right miRNAs are involved, respectively, in either predominant biological pathways of left- or right-sided CRC (Table III). Earlier studies have found that the Wnt, (7) mTor, and PI3K-Akt signaling pathways are predominant in left-sided CRC (4, 5). We identified that the participation of left miRNAs in these pathways was significant (FDR<0.05), whereas the right miRNAs were more prevalent than left ones in the TGF-β pathway ($FRD=6.24\times10^{-6}$ <FRD=0.007). This pathway has been previously observed together with MSI in right-sided CRC (21). We saw no TGF-β pathway-MSI connection in the pathway analysis, but the MSI cases were more frequent in the right CRC samples compared to the left samples in both cohorts (Table I). Earlier reports support our findings of predominant biological pathways in site-specific CRC, however, our novel observation is that miRNAs exhibit biological pathway specificity.

We studied whether the identified predominant biological pathways of the left and right miRNAs in the discovery cohort could be confirmed in the validation cohort. Only the participation of left miRNAs in the Wnt signaling pathway (dominance in left-sided CRC) was significant (FDR<0.05) in both cohorts (Table III). Other pathways *e.g*., the mTor and MAPK signaling pathways, with the left and the right miRNAs, were significant (FDR<0.05) in the validation cohort but showed no similarities in the discovery cohort. Furthermore, the TGF-β signaling pathway was discordant in the validation cohort compared to the discovery cohort, as the association of this pathway with the left miRNAs was much more significant ($FDR=6.15\times10^{-10}$) than that with the right miRNAs (FDR=0.004). The pathway differences between the cohorts may be influenced by the characteristics of the samples. The discovery and validation tumor samples differ slightly in the tumor progression level. Some (13%) of the validation tumor samples were further advanced (stage IV-IVB), whereas none of the discovery tumor samples were in this stage (Table I). Microenvironmental factors such as surrounding blood vessels, immune cells, signaling molecules and extracellular matrix vary in different tumor stages. This may influence the transcription of cancer-related genes; thus, the expression of miRNAs can vary in different tissue microenvironments (22, 23). This could particularly affect cancer-related miRNAs and result in easier detection of high expression levels of them. Despite the possible heterogeneity

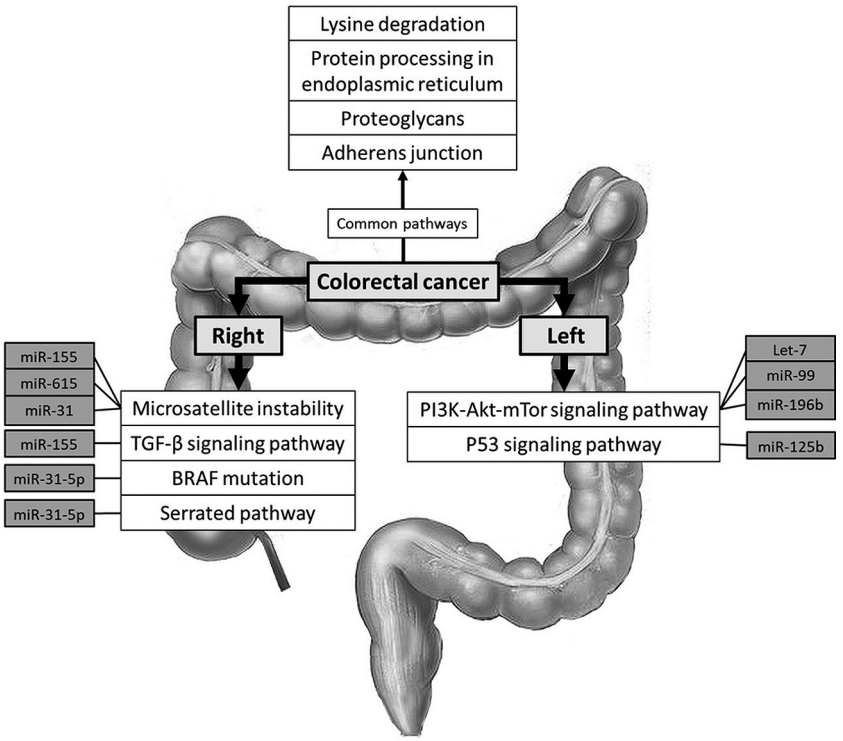

Figure 1. *MiRNAs and predominant biological pathways in CRC. The left and right miRNAs from our discovery and validation cohorts regulate common pathways in CRC. Individual miRNAs may relate to predominant biological pathways of left- and right-sided CRC. CRC: Colorectal cancer; miRNA: microRNA.*

in the tissue microenvironment of the samples, we observed that the left and right miRNAs of both cohorts participated in four shared biological pathways (FDR<1×10^{-5}) (Figure 1). This indicates that the left and right miRNAs participate together in common processes in CRC.

Our top (FDR<0.01) left (let-7, miR-99 and miR-125b) and right (miR-155 and miR-615) miRNAs may be site-specific miRNAs (Table S8) as they regulate the predominant biological pathways of site-specific CRC (Figure 1). The P13K/Akt/mTor signaling pathway is prevalent in left-sided CRC (4, 5). Let-7 (24) and miR-99 (25) are involved in the regulation of this pathway indicating that these miRNAs can be prevalent in left-sided CRC. The *TP53* mutation is predominant in left-sided CRC, (3) and miR-125b regulates the p53 protein levels (26). This suggests that the p53 signaling pathway is important in left-sided CRC. However, in our analysis, both left (including miR-125b) and right miRNAs were significantly (FDR<0.05) involved in the p53 signaling pathway (Table III). In right-sided CRC, MSI is predominant compared to left-sided CRC (7). Our right miR-155 (27) and miR-615 (28) may play a role in MSI, as indicated by their up-regulation in right-sided CRC.

Altogether, six shared DE miRNAs were observed in both cohorts. A small sample size of the discovery cohort (N=24) presented a limitation to the statistical power of the analyses and may partly explain the low amount of shared common miRNAs. In the future, a larger number of left- and right-sided CRC samples is needed to confirm our results. However, among the shared six miRNAs, three (hsa-miR-196b-5p, hsa-miR-625-3p and hsa-miR-155-5p) were significantly (FDR<0.05) related to the CRC pathway (Table IV). Some of the shared miRNAs may have a prominent role in predominant biological pathways of site-specific CRC (Figure 1 and Table S8). We propose that miR-196b may be predominant in left-sided CRC by regulating the PI3K/Akt/mTor pathway (4, 5, 29). Further, miR-196b has been reported earlier as up-regulated in left-sided CRC (30). We suggest that miR-31-5p may be dominant in right-sided CRC as it has been linked to MSI (31). Moreover, miR-31-5p has been previously connected with the *BRAF* mutation (32) and serrated pathways, which are predominant in right-sided CRC (6).

Our findings may be beneficial not only to the classification of CRC or biomarker studies on the early detection of site-specific CRC but also for treatment development. The fact that with right-sided CRC the prognosis is poorer than with left-sided CRC may be partially explained by biological pathways affecting only right-sided CRC (2, 7). For example, *BRAF* mutation is

predominant in right-sided CRC, and this mutation is associated with a poorer outcome of metastatic CRC patients (33). Also, *BRAF*^{V600E} mutation and MSI-H are associated with the serrated pathway (34), which also predicts a poor outcome in right-sided CRC (7). The understanding of the predominant biological pathways in CRC advances drug development, *e.g.*, currently, the BRAF^{V600E} inhibitor is in clinical testing also for CRC (33). Additionally, miRNAs related to predominant pathways can be considered as biomarkers for site-specific CRC. For example, in our data, miR-31-5p was up-regulated in right-sided CRC (Table II) and also others have suggested this miRNA as a therapeutic biomarker for CRC patients with a *BRAF* mutation (6).

The poor prognosis of right-sided CRC may be also explained by the characterization of miRNAs. For example, four miRNAs (miR-10b, miR-155, miR-625-3p and miR-31), which were our "right miRNAs", have been shown to enhance CRC progression (35-38). Up-regulation of miR-10b was associated with tumor invasion, advanced tumor stage and liver metastasis in CRC. High expression level of miR-10b was related to poor prognosis in both early stage and advanced forms of CRC (35). Overexpression of miR-155 (36) and miR-625-3p (37) promotes migration and invasion, and overexpression of miR-31 (38) advances proliferation in CRC cells.

To conclude, our findings add to the existing literature regarding different miRNA expression profiles between left- and right-sided CRC. We found that *e.g.*, miR-23a-3p, miR-193b-3p, miR-218-1-3p, mR-9-5p and miR-3074-5p were up-regulated in left-sided CRC and *e.g.*, miR-589-5p, miR-330-5p, miR-942-5p, hsa-miR-15a-5p and miR-130b-5p were up-regulated in right-sided CRC (Table II). Furthermore, we also propose that some individual miRNAs, by regulating predominant biological pathways, may be prevalent in site-specific CRC (Figure 1). Because our findings are preliminary, our site-specific miRNAs and their suggested predominant biological pathways should be tested with *in vivo* and *in vitro* experiments to gain clinical importance. Our findings may give new insights for classifying left- and right-sided CRC and enable the identification of potential miRNA biomarkers for the two forms of CRC.

Conflicts of Interest

The Authors have declared no conflicts of interest regarding this study.

Authors' Contributions

S.H., J.M.H., T.K., J.-P.M., V.-M.K. and A.M. conceived and planned the experiments. T.K. and J.-P.M. contributed to collecting the samples. S.E. and J.M.H. prepared the samples for RNA sequencing. J.M.H. performed RNA sequencing. S.H. contributed to the bioinformatics data analysis. S.E. contributed to the biological pathway analysis. S.E., S.H., J.M.H., T.K. and A.M. contributed to the interpretation of the results. S.E. wrote the manuscript with support from S.H., J.M.H., T.K. and A.M. All Authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Supplementary material

The supplementary material is available online at: https://zenodo.org/record/3887948#.XuuI9mgzZPY

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