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Cancer Epidemiol Biomarkers Prev. Author manuscript; available in PMC 2024 March 01.

Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2023 September 01; 32(9): 1146–1152. doi:10.1158/1055-9965.EPI-22-1020.

Elevated *EVL* methylation level in the normal colon mucosa is a potential risk biomarker for developing recurrent adenomas

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Abstract

Background: Individuals with adenomatous colorectal polyps undergo repeated colonoscopy surveillance to identify and remove metachronous adenomas. However, many patients with adenomas do not develop recurrent adenomas. Better methods to evaluate who benefits from increased surveillance are needed. We evaluated the use of altered *EVL* methylation as a potential biomarker for risk of recurrent adenomas.

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Conflict of Interest and Financial Disclosure Statements: W. M. Grady is an advisory board member for Freenome, Guardant Health, and SEngine and consultant for DiaCarta, Natera, Helio, Guidepoint and GLG. He is an investigator in a clinical trial sponsored by Janssen Pharmaceuticals and receives research support from Tempus and LucidDx. R.E. Schoen receives research support from Freenome, Exact Sciences, and Immunovia. C.M. Ulrich has as cancer director oversight over research funded by several pharmaceutical companies but has not received funding directly herself.

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Methods: Patients with 1 colonoscopy had EVL methylation (*mEVL*) measured with an ultra-accurate methylation-specific droplet digital PCR assay on normal colon mucosa. The association between *EVL* methylation levels and adenoma or colorectal cancer (CRC) was evaluated using three case/control definitions in three models: unadjusted (model 1), adjusting for baseline characteristics (model 2), and an adjusted model excluding patients with CRC at baseline (model 3).

Results: Between 2001 and 2020, 136 patients were included; 74 healthy patients and 62 patients with a history of CRC. Older age, never smoking and baseline CRC were associated with higher levels of m*EVL* (p 0.05). Each log^{10} difference in mEVL was associated with an increased risk of adenoma(s) or cancer at/after baseline for model 1(OR: 2.64 95% CI: 1.09–6.36), and adenoma(s) or cancer after baseline for models 1(OR: 2.01, 95% CI: 1.04–3.90) and model 2 (OR: 3.17, 95% CI: 1.30–7.72).

Conclusion: Our results suggest that *EVL* methylation level detected in the normal colon mucosa has the potential to be a biomarker for monitoring the risk for recurrent adenomas.

Impact: These findings support the potential utility of *EVL* methylation for improving the accuracy for assigning risk for recurrent colorectal adenomas and cancer.

Keywords

DNA methylation; colorectal cancer; field cancerization effect; risk biomarkers; droplet digital PCR; metachronous adenomas

Introduction

Colorectal cancer (CRC) is the fourth most common cancer and the second leading cause of cancer-related death in the United States.(1) The majority of CRC arises from pre-cancerous adenomas. Randomized trials demonstrate that removal of these pre-cancerous lesions is associated with reduced subsequent CRC incidence.(2,3) Individuals with adenomas, especially those with advanced adenomas, are at heightened long-term risk of subsequent CRC compared to those with no personal history of adenoma.(4–7) As such, individuals with a history of colon adenomas are classified as higher risk and advised to undergo more frequent surveillance colonoscopy exams compared to people with no history of colon adenomas or cancer.(8) However, with an approximate 10% recurrence rate within 3–5 years(9), more frequent surveillance colonoscopy in all of these individuals is an overly expensive and inefficient approach to decrease CRC incidence. Alternative means of determining risk beyond what is currently used are being explored, such as development of polygenic and environmental factor risk scores.(10,11) Few studies have evaluated the use of molecular markers to accurately predict an individual's risk of metachronous adenomas or cancer during surveillance colonoscopy.

The increased risk of adenomas and cancer in some people may be partly related to a field cancerization phenomenon, or 'field effect', in the normal colon that predisposes the colon to develop adenomas or CRC.[(12),(13)] Field cancerization was originally assumed to involve relatively small (few centimeters from a neoplastic lesion) regions, but more recent studies suggest that the field may involve the entire organ.(14,15) Molecular changes in these areas

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of field cancerization have the potential to serve as biomarkers to identify individuals at high risk for developing adenomas or CRC.

Aberrantly methylated genes are found in virtually all colon adenomas and CRC and are common even in early adenomas. They hold promise as risk biomarkers for CRC, possibly through a role in field cancerization.(16,17) For example, methylated *EVL* (gene name: Ena Vasp Like) and other genes have been detected at higher frequency in the normal colon of people with CRC compared to average risk individuals, which may reflect a field cancerization process.(16,18,19) However, because of the scarcity of tissue samples and appropriate cohorts, the potential of methylated genes to be used as CRC risk biomarkers has not been evaluated for predicting the risk of metachronous adenomas. This is also due to the lack of sensitive and precise detection methods for methylated genes, since, based on our current understanding of field cancerization, the methylated alleles present in normal colon mucosa in the setting of field cancerization are present at levels that would be expected to be below the detection limits of current PCR technologies. Recently, a more precise and sensitive method has been developed to detect low levels of methylated DNA based on droplet digital PCR technology, which can more accurately determine whether methylated genes can be used as field effect markers.(20,21) In this study, based on our previously published studies, which showed *mEVL* commonly in the normal colon of people with CRC but not in cancer free people (16), we aimed to evaluate whether aberrant *EVL* methylation in normal colonic mucosa is associated with the risk of developing metachronous adenomas in a well-characterized patient cohort with long-term follow-up.

Materials and Methods

Study Population and Definitions

The study population was a retrospective cohort of patients at the University of Washington Medical Center (UWMC, Seattle, Washington, USA) who underwent colonoscopy for colorectal cancer screening or surveillance combined with patients with a history of CRC who underwent surveillance colonoscopy. To be included, patients had to have the results of at least one colonoscopy as well as a tissue sample (biopsy) from the normal colon mucosa to assess methylated *EVL* levels (m*EVL*) levels. Patients were selected from the GICaRes and ColoCare studies (22,23). (Supplemental Figure 1) All studies were done following protocols approved by the institutional IRB committees (23). The GICaRes and CoCare staff obtained written informed consent from the study subjects. The studies were conducted in accordance with the Belmont Report, Declaration of Helsinki and US Common Rule.

Sample Acquisition and Preparation

Tissue samples of normal colon mucosa collected at the University of Washington Medical Center were collected by endoscopic biopsy from patients undergoing screening or surveillance colonoscopies or by surgical resection at the time of CRC diagnosis. To avoid the potentially confounding effects of anatomic location, only samples from the left colon (defined as distal to the hepatic flexure) were included in the study. Tissue samples were snap frozen in liquid nitrogen and transferred to a −80°C freezer for long-term storage.

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Genomic DNA Extraction and Quantification

DNA was extracted from tissue samples using the DNeasy® Blood and Tissue Kit (QIAGEN, catalog #69504). DNA concentration was measured using QuantiT™ PicoGreen® dsDNA Assay Kit (Life Technologies, catalog #p7589)

Bisulfite Conversion of gDNA

DNA Samples (100ng) were bisulfite converted using the EZ DNA Methylation Kit (ZymoResearch Cat #D5002) following the manufacturer's instructions. The bisulfite-converted samples were eluted in a 20-uL volume and stored at −80°C until needed.

Methylation-specific ddPCR (MS-ddPCR)

The MS-ddPCR reaction mixture consisted of the 2X ddPCR Supermix for Probes No dUTP (BioRad Cat #186–3024), and locus specific primers and probes. The primer and probe sequences for methylated *EVL* (NP_057421) were designed using ABI PrimerExpress software Version 5.0.17 (Applied Biosystems, Life Technologies), and synthesized with a FAM reporter. (Supplemental Table 1) We determined the relative amounts of each sample through a C-LESS-C1 assay, which amplifies the total amount of DNA in a PCR reaction. The C-LESS probe was synthesized with a VIC reporter. The primer and probes were used at final concentrations of 900 and 250 nmol/L, respectively. Various amounts of bisulfite-converted DNA were used in a final volume of 20 μL. Each 20-μL PCR reaction was loaded into the Bio-Rad DG8 disposable droplet generation cartridge (BioRad, Hercules, CA). A volume of 70μL of droplet generation oil was loaded into adjacent oil wells. The microfluidic chip was loaded into a droplet generator (BioRad, Hercules, CA). The resulting water-in-oil droplets were pipette-transferred from the outlet well to a 96-well polypropylene plate. The plate was heat-sealed with PX1™ PCR Plate Sealer (BioRad, Hercules, CA), placed on a T100TM Thermal Cycler (BioRad, Hercules, CA) and amplified to the endpoint. The thermal cycling conditions were 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min (2.5°C/s ramp rate) with a final 10 min hold at 98°C. After PCR amplification, the 96-well PCR plate containing droplets was loaded into a QX200 droplet reader (Bio-Rad). The ddPCR system partitions the 20 μL PCR reaction into an average of 15,000 nanoliter droplets and each droplet from each well of the plate was read with a 2-color fluorescence reader to determine how many droplets were positive for the methylated *EVL* (in FAM), as well as for the control reaction C-LESS-C1(in VIC). All methylation quantification experiments included no-template-controls (NTC) wells, which contained all the components of the reaction except for the DNA template, control wells containing 2,500pg of 100% methylated EpiTect Methyl control DNA (QIAGEN Cat #59655) and 2,500 pg of 100% unmethylated EpiTect Unmethyl control DNA (QIAGEN Cat #59665). No amplification signal was detected in NTC wells. Data was analyzed using the QuantaSoft software version 1.4.0.99 (BioRad, Hercules, CA). The *EVL* methylation level was measured as the ratio: number of methylated *EVL* droplets divided by the number of positive C-LESS droplets within each sample, expressed as percentage.

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Case and Control Definitions for Analyses

Since the schedule of follow-up colonoscopies was not pre-specified, patients had differing numbers of follow-up colonoscopies and durations of follow-up to address the question of this work: are m*EVL* levels predictive of development of adenoma(s)? To evaluate the association between m*EVL* levels in normal colon and the detection of adenoma(s) at the initial colonoscopy or follow-up colonoscopy (termed metachronous or recurrent adenoma(s)), we defined three major comparison sets using different definitions of a "control" and "case" group. The first comparison set (Comparison Set 1) defined the control group as patients without adenoma(s) or cancer detected at baseline or any follow-up colonoscopy and the case group as those with any adenoma(s) or cancer detected at baseline or any follow-up colonoscopy. The second comparison set (Comparison Set 2) was restricted to patients with at least one follow-up colonoscopy. The control group included patients with neither adenoma(s) nor cancer detected after baseline at any follow-up exam and the case group included patients with an adenoma(s) or cancer detected after baseline on a follow-up colonoscopy. The third comparison set (Comparison Set 3) defined the control group as patients with at least 4 years of follow-up after baseline and no adenoma(s) or cancer detected during follow-up. The case group is the same as for Comparison Set 2. For Comparison Sets 2 and 3, patients with insufficient follow-up were excluded. These definitions of control and case groups for the three comparison sets are also included in the tables.

Statistical Analysis

A linear regression model was used to evaluate the unadjusted association between $\log_{10}$ transformed m*EVL* and baseline characteristics. Next, logistic regression models were used to evaluate the association between case/control status and $\log_{10}$ *EVL* methylation levels for the three comparison sets. Within each comparison set three analyses were performed. The first analysis evaluated the unadjusted association, the second analysis repeated this comparison but also adjusted for age, sex, smoking status, adenoma(s) at baseline colonoscopy (excluded from Comparison Set 1), and cancer at baseline colonoscopy (excluded from Comparison Set 1), and the third analysis excluded patients with CRC at their baseline colonoscopy and was adjusted for age, sex, smoking status, and presence of adenoma(s) at baseline colonoscopy (excluded from Comparison Set 1). Statistical significance was based on a two-sided alpha level of 0.05. SAS Version 9.4 was used for all statistical analyses.

Data Availability

The data generated in this study are available upon request from the corresponding author.

Results

Between 2001 and 2020, 136 patients seen at UWMC met the criteria to be included in the analysis (Figure 1, Supplemental Figure 1). Among them were 74 healthy patients who underwent colonoscopy for CRC screening or surveillance and 62 patients with a history of CRC who underwent surveillance colonoscopy. The majority were male (55%), white (83%), never smokers (63%), and the median age at initial colonoscopy was 54

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years (range: 29, 85). Just over half (51%) had the results of three or more colonoscopies. Fifty-four percent of patients had adenoma(s) and 48% had cancer detected at the initial colonoscopy. (Supplemental Tables 2–4) As described in Table 1, the median and range of *EVL* methylation levels were 0.17 (0, 6.89).

Association between m*EVL* levels and patient characteristics

Table 2 presents the association between baseline characteristics and $\log_{10}$ transformed m*EVL* levels. Older age and having cancer at baseline were associated with higher levels of m*EVL* ($p<0.001$ and $p=0.05$, respectively). Being an ever or current smoker compared to a never smoker was associated with lower levels of m*EVL* ($p = 0.01$). Sex, body mass index, and presence of adenoma(s) at baseline were not associated with m*EVL* levels ($p>0.05$ for all).

Association between m*EVL* levels and detection of adenoma(s) or cancer at baseline or follow-up

For Comparison Set 1, 14 patients never had adenoma(s) or cancer detected at the baseline or any follow-up examinations and 122 were had adenoma(s) or cancer detected at baseline or during their follow-up period (Figure 2, **panel A**). The association between m*EVL* levels and case/control status for the Comparison Set 1 analyses are presented in Table 3. (Supplemental Figure 2) The univariable model estimates a 2.64-fold increased risk of ever having adenoma(s) or cancer detected for each one-log increase in m*EVL* levels ($p=0.03$). The multivariable model estimates a 1.92-fold increased risk of adenoma(s) or cancer detection adjusting for potential confounding factors ($p=0.22$). The multivariable model excluding patients with cancers estimates a 1.97-fold increased risk of adenoma(s) detection ($p=0.27$). (Supplemental Table 5)

Association between m*EVL* levels and detection of adenoma(s) at follow-up

Comparison Set 2 evaluated the association between m*EVL* levels and detection of adenomas and/or cancer after the initial colonoscopy. For this set of analyses, 43 patients never had adenoma(s) or cancer detected on a follow-up examination, 71 had adenoma(s) detected during their follow-up period, and 22 patients had insufficient follow-up and were not included in this set of analyses (Figure 2, **panel B,** Supplemental Figure 3).

The results of the three models for Comparison Set 2 are in Table 3. The univariable model estimates a 2.01-fold increased risk of detecting adenoma(s) or cancer for each $\log_{10}$ increase in m*EVL* levels ($p=0.04$), the multivariable model estimates a 3.17-fold increased risk of adenoma(s) or cancer detection adjusting for potential confounding factors ($p=0.01$), and the multivariable model excluding patients with cancers estimates a 3.75-fold increased risk of adenoma(s) ($p=0.07$). (Supplemental Table 6)

Association between m*EVL* levels and detection of adenoma(s) with a minimum of 4 years of follow-up

Comparison Set 3 uses a stricter follow-up definition for inclusion in the control group and the same definition for the case group as for Comparison Set 2. For Comparison Set 3, 25 patients never had adenoma(s) or cancer detected and had at least 4 years of follow-up,

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71 had adenoma(s) detected during their follow-up period, and 40 patients had insufficient follow-up and were not included in this set of analyses (Figure 2, **panel C,** Supplemental Figure 4).

The results of the three models for Comparison Set 3 are presented in Table 3. The univariable model estimates a 2.29-fold increased risk of ever having adenoma(s) or cancer detected for each log increase in m*EVL* levels (p=0.05), the multivariable model estimates a 2.77-fold increased risk of adenoma(s) or cancer detection adjusting for potential confounding factors (p=0.09), and the model excluding patients with cancers, estimates a 3.08-fold increased risk of adenoma(s) (p=0.14). (Supplemental Table 7)

Discussion

Colorectal cancer (CRC) arises from the accumulation of genetic and epigenetic alterations in colon epithelial cells that drive adenoma to cancer progression. Epigenetic alterations, including aberrant DNA methylation, are the earliest molecular changes that arise in incipient cancers, with hundreds to thousands of aberrantly methylated loci commonly found in colon adenomas.(24) They are also observed in the normal colon of people at increased risk for CRC, including those with a personal history of CRC and in people with ulcerative colitis.(16,25) These observations have raised the possibility that aberrant DNA methylation in morphologically normal colon mucosa may be an early molecular alteration in normal colon cells that can lead to the initiation and progression of CRC.

In light of our findings that methylated *EVL* was present at higher frequency in the normal colon of people with CRC compared to average risk individuals, suggesting that it may indicate a field cancerization state(16), we developed a quantitative methylation-specific ddPCR (MS-ddPCR) assay, which allows for a highly precise and sensitive quantification of methylated alleles to examine methylated *EVL* in the normal mucosa samples from patients with CRC.(20,21) In this study, we used a unique sample set with detailed clinical annotation and state of the art DNA methylation detection methods to evaluate the potential to use molecular markers for predicting the risk of metachronous lesions. We observed increased m*EVL* in older patients and decreased *mEVL* in tobacco users. This finding is consistent with previously published studies that found alterations in DNA methylation patterns in the elderly due to epigenetic aging(26,27) but in contrast to studies that have shown increased gene methylation, including *AHRR, ALDH3A1, CYP1A1,* and *CYP1B1,* in tobacco users (28). These observations raise the possibility that increased *mEVL* may be secondary to the DNA methylation alterations observed with biological aging (26,27).

With regards to the potential for *mEVL* to be a risk marker for recurrent colon adenomas, compared with patients who never had adenoma(s) or cancer detected on a follow-up examinations (Comparison Set 2), the univariable model estimates a 2.01-fold increased risk of detecting adenoma(s) or cancer for each $\log_{10}$ increase in m*EVL* levels (95% CI: 1.04–3.90, p=0.04), the multivariable model estimates a 3.17-fold increased risk of adenoma(s) or cancer detection adjusting for potential confounding factors (95% CI: 1.30–7.72, p=0.01), and the multivariable model excluding patients with cancer at baseline estimates a 3.75-fold increased risk of adenoma(s) (95% CI: 0.91– 15.49, p=0.07). These findings support the

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potential utility of this molecular marker to identify people at elevated risk for recurrent colon adenomas and may be appropriate for enhanced colon cancer surveillance or for chemoprevention therapies.

It is noteworthy that our study has certain limitations that may have affected our results. First, it is a retrospective study and subject to unrecognized confounding factors. There was not a regimented interval between colonoscopies, so the differences between follow-up intervals may have affected our results, although we attempted to correct for this in Comparison Set 3 by requiring at least 4 years of follow up. Furthermore, we assumed that *EVL* methylation levels are constant throughout the observation period regardless of when the sample was collected within the study timeframe, but this has not been proven for *mEVL* status. Another factor that could have influenced DNA methylation analysis is the tissue source. Our study included normal colon mucosa samples from both endoscopic biopsy of patients undergoing colonoscopies and surgical resection specimens from patients with a history of CRC. The ultrasensitive MS-ddPCR assay enabled the absolute quantification of *EVL* methylation in both sample types, thus minimizing the tissue source variable, but may not have completely removed it(21). Importantly, we did account for the variability of *EVL* methylation in the normal colon based anatomical location in the colon(29) by only including samples from the left colon in our study. We also recognize that our study has a relatively small sample size, particularly for patients with more than one follow-up colonoscopy, though it is the largest such sample set with longitudinal and colonoscopy data available to our knowledge.

In summary, our results suggest that the *EVL* methylation level in the normal colon mucosa has potential to be used as a risk biomarker for recurrent adenomas. These results support future studies in an independent study with a prospective design to evaluate the potential of *EVL* methylation as a CRC risk biomarker to be used to individualize clinical colorectal cancer prevention programs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We wish to acknowledge and thank the outstanding contributions by the ColoCare team and Kathy Vickers, the University of Washington GiCaRes Translational Research Team (Wynn Burke, Brian Foerster, Colton Johnson, Lucilla Bella and Joselito Morada), the UW Digestive Health Center staff, and Grady lab members.

FUNDING

National Cancer Institute, Grant/Award Number: R50CA233042 to M. Yu; National Institutes of Health, Grant/Award Number: U54CA274374, UO1CA152756, RO1CA220004, UO1AG077920; Listwin Family Foundation; Cottrell Family Fund; R.A.C.E. Charities to W.M. Grady. Supported by National Institutes of Health Early Detection Research Network U01-CA152753 (R.E. Schoen). J. Ose, B. Gigic, J.C. Figueiredo, A.T. Toriola, E.M. Siegel, C.I. Li, A. Ulrich, M. Schneider, D. Shibata, and C.M. Ulrich received funding by the NIH U01CA206110, NIH R01CA207371 and NIH R01189184. C.M. Ulrich is supported by NIH R01 CA211705. C.M. Ulrich received funding by the Huntsman Cancer Foundation.

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List of Abbreviations:

CRC	colorectal cancer
m*EVL*	methylated *EVL*
PCR	polymerase chain reaction
ddPCR	droplet digital PCR
CV	coefficients of variation
LOQ	limit of quantification
LOD	limit of detection
NTC	no-template control

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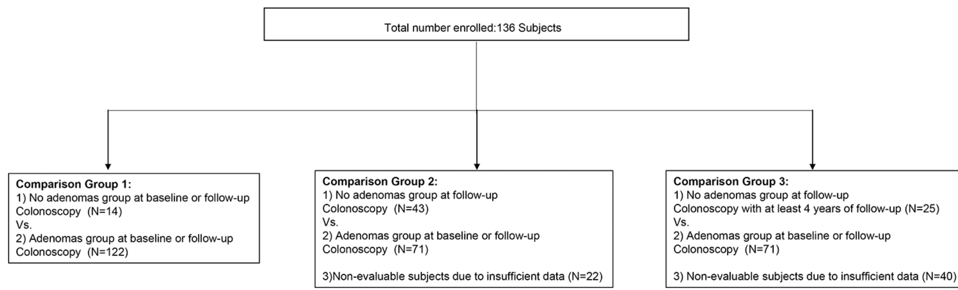

Figure 1:

Diagram of patients included in the different comparison group analyses described in the Methods and Results sections. One hundred and thirty-six subjects were assessed and compared in Comparison Groups 1–3 as shown.

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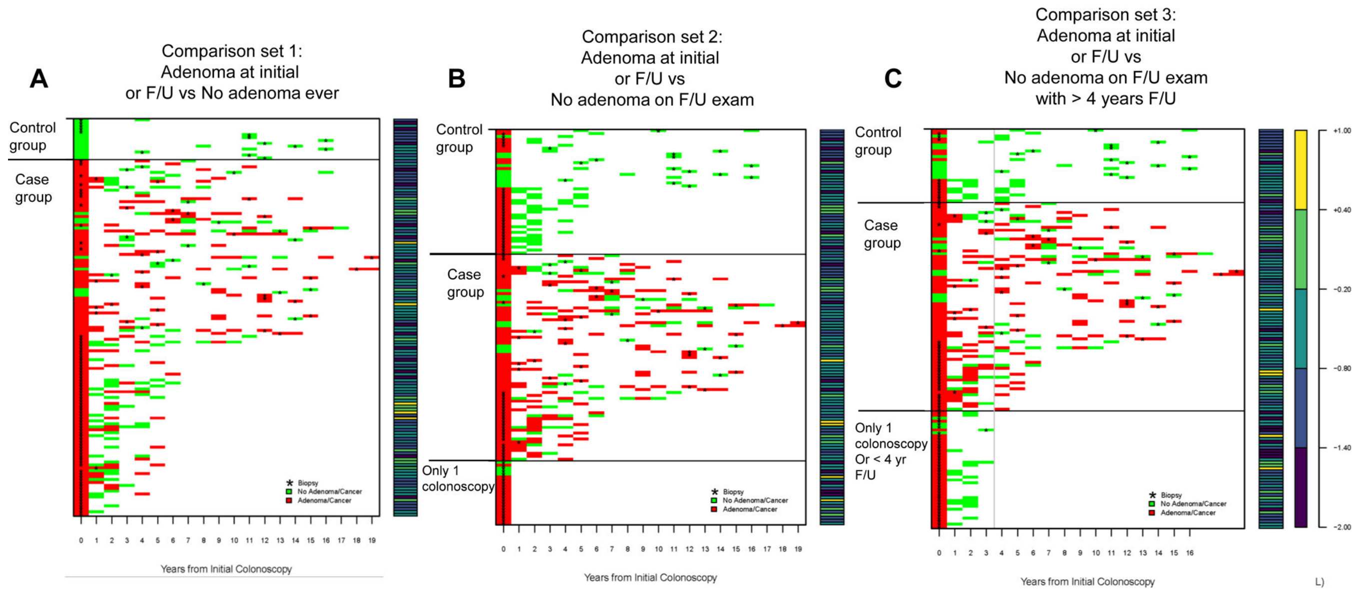

Figure 2:

Graphical representation of patient information on timing and outcome of colonoscopies for patients included in the three sets of comparisons. **(A) Comparison Set 1:** any adenoma(s) or cancer ever vs. no adenoma(s) or cancer ever **(B) Comparison Set 2:** adenoma(s) or cancer(s) detected at follow up vs. no adenoma(s) at follow up; **(C) Comparison Set 3:** adenoma(s) or cancer(s) detected at follow up vs. no adenoma(s) at least 4 years of follow up. Each row represents one patient, and each rectangle represents a colonoscopy examination. The colonoscopy timing, results of the colonoscopies, and timing of the biopsies are shown. The side panel displays a heatmap of the *EVL* methylation levels for each patient. **Green**: no adenoma(s) detected; **Red**: adenoma(s); **White**: no colonoscopy at timepoint; * indicates timepoint tissue biopsy was taken for DNA extraction and *EVL* methylation measurement.

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Table 1:

Patient Characteristics

	N = 136*
	n (%)
Age at initial Colonoscopy, *Median (Range)*	54.3 (28.5, 85.0)
Age at Tissue Biopsy, *Median (Range)*	58.3 (28.5, 85.0)
Male Sex	75 (55%)
Race	
White	113 (83%)
Black	4 (3%)
Asian	10 (7%)
Multiple	4 (3%)
Unknown	5 (4%)
Body Mass Index (BMI)	27.8 (17.8, 67.0)
Smoking History	
Never Smoker	63 (50%)
Former Smoker	32 (25%)
Current Smoker	31 (25%)
Adenoma(s) at the initial Colonoscopy	73 (54%)
Cancer at the Colonoscopy	65 (48%)
Total # of Colonoscopy Visits	
Median (Range)	3 (1, 9)
1	22 (16%)
2	45 (33%)
3+	69 (51%)
Years of Follow-Up, *Median (Range)*&	4.2 (0.6, 19)
%m*EVL*, *Median (Range)*	0.17 (0.00, 6.89)
Log_{10}(%m*EVL*), *Median (Range)*	−0.74 (−2.00, 0.84)

*BMI (n=125), Smoking Status (n=126),

&Among patients with at least one follow-up visit (n=114)

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Table 2:

Association between initial characteristics and log10 m*EVL* levels

Variable	Linear Regression Parameter (95% Confidence Interval)	P-value
Age at Tissue Biopsy	0.025 (0.015, 0.034)	< 0.0001
Sex		
Male (vs. Female)	0.138 (−0.079, 0.355)	0.211
Smoking History		
Current/Former (vs. Never)	−0.274 (−0.488, −0.059)	0.013
Body Mass Index (BMI)	0.003 (−0.013, 0.020)	0.693
Adenoma at initial colonoscopy		
Yes (vs. No)	0.005 (−0.213, 0.223)	0.965
Cancer at initial colonoscopy		
Yes (vs. No)	0.214 (0.000, 0.429)	0.050
Race White (vs. Non-White)	−0.079 (−0.400, 0.243)	0.630

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Table 3:

Model Results Evaluating the Association between $\log_{10}$ m*EVL* Levels and Risk of Adenoma(s) or Cancer

Comparison Set	Case Group	Control Group	Odds Ratio (95% Confidence Interval), p-value		
			Univariable Models	Multivariable Models[1]	Multivariable Models excluding cancers at initial colonoscopy [2]
1	Any Adenoma(s) or Cancer detected at initial or follow-up colonoscopy	No adenoma(s) or cancer detected at initial or follow-up	n= 136 2.64 (1.09,6.36) p = 0.03	n= 126 1.92 (0.68,5.41) p = 0.22	n= 67 1.97 (0.59,6.53) p = 0.27
2	Adenoma(s) or Cancer detected after initial colonoscopy	No adenoma(s) or Cancer detected after initial	n= 114 2.01 (1.04,3.90) p = 0.04	n= 110 3.17 (1.30, 7.72) p = 0.01	n= 60 3.75 (0.91, 15.49) p = 0.07
3	Adenoma(s) or Cancer detected after initial colonoscopy	No adenoma(s) or cancer detected after initial with at least 4 years follow-up	n= 96 2.29 (0.99,5.3) p = 0.05	n= 93 2.77 (0.87, 8.85) p = 0.09	n= 58 3.08 (0.69,13.79) p = 0.14

[1] Models adjusted for age at initial colonoscopy, sex, smoking status in **Comparison Set 1**; Models adjusting for age at initial colonoscopy, sex, smoking status, adenoma(s) at initial colonoscopy, cancer at initial colonoscopy for **Comparison Sets 2** & **3**.

[2] Models adjusting for age at 1st colonoscopy, sex, smoking status for **Comparison Set 1**; Models adjusting for age at initial colonoscopy, sex, smoking status, adenoma(s) at initial colonoscopy for **Comparison Sets 2** & **3**.