The Journal of Molecular Diagnostics, Vol. 24, No. 6, June 2022

ELSEVIER

the Journal of Molecular Diagnostics
jmdjournal.org

Epigenome-Wide DNA Methylation Profiling of Normal Mucosa Reveals *HLA-F* Hypermethylation as a Biomarker Candidate for Serrated Polyposis Syndrome

Gerhard Jung,[*†‡§] Eva Hernández-Illán,[†] Juan J. Lozano,[‡¶] Julia Sidorova,[‡¶] Jenifer Muñoz,[†‡] Yasuyuki Okada,[‖**] Enrique Quintero,[††] Goretti Hernandez,[††] Rodrigo Jover,[‡‡] Sabela Carballal,[*†‡§] Miriam Cuatrecasas,[†‡§,§§] Lorena Moreno,[*†‡§] Mireia Diaz,[*†‡§] Teresa Ocaña,[*†‡§] Ariadna Sánchez,[*†‡§] Liseth Rivero,[*†‡§] Oswaldo Ortiz,[*†‡§] Joan Llach,[*†‡§] Antoni Castells,[*†‡§] Maria Pellisé,[*†‡§] Ajay Goel,[‖¶¶] Eduard Batlle,[‖‖] and Francesc Balaguer[*†‡§]

From the Gastroenterology Department and the Pathology Department,[§§] Hospital Clínic de Barcelona, Barcelona, Spain; the Institut d'Investigacions Biomèdiques August Pi i Sunyer,[†] Barcelona, Spain; the Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD),[‡] Barcelona, Spain; the Faculty of Medicine,[§] University of Barcelona, Barcelona, Spain; the Bioinformatics Platform,[¶] CIBEREHD, Barcelona, Spain; the Department of Molecular Diagnostics and Experimental Therapeutics,[‖] Beckman Research Institute, Biomedical Research Center, Monrovia, California; the Department of Gastroenterology and Oncology,** Tokushima University Graduate School, Tokushima, Japan; the Department of Gastroenterology,[††] University Hospital of the Canary Islands, Santa Cruz de Tenerife, Spain; the Servicio de Medicina Digestiva,[‡‡] Hospital General Universitario de Alicante, Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL), Alicante, Spain; the City of Hope Comprehensive Cancer Center,[¶¶] Duarte, California; and the Institute for Research in Biomedicine (IRB Barcelona),[‖‖] The Barcelona Institute of Science and Technology, Barcelona, Spain*

Accepted for publication March 4, 2022.

Address correspondence to Francesc Balaguer, M.D., Ph.D., Hospital Clínic, Carrer Villarroel 170, 08036 Barcelona, Spain.
E-mail: fprunes@clinic.cat.

Serrated polyposis syndrome (SPS) is associated with a high risk for colorectal cancer. Intense promoter hypermethylation is a frequent molecular finding in the serrated pathway and may be present in normal mucosa, predisposing to the formation of serrated lesions. To identify novel biomarkers for SPS, fresh-frozen samples of normal mucosa from 50 patients with SPS and 19 healthy individuals were analyzed by using the 850K BeadChip Technology (Infinium). Aberrant methylation levels were correlated with gene expression using a next-generation transcriptome profiling tool. Two validation steps were performed on independent cohorts: first, on formalin-fixed, paraffin-embedded tissue of the normal mucosa; and second, on 24 serrated lesions. The most frequently hypermethylated genes were *HLA-F*, *SLFN12*, *HLA-DMA*, and *RARRES3*; and the most frequently hypomethylated genes were *PIWIL1* and *ANK3* ($\Delta\beta = 10\%$; $P < 0.05$). Expression levels of *HLA-F*, *SLFN12*, and *HLA-DMA* were significantly different between SPS patients and healthy individuals and correlated well with the methylation status of the corresponding differentially methylated region (fold change, >20%; $r > 0.55$; $P < 0.001$). Significant hypermethylation of CpGs in the gene body of *HLA-F* was also found in serrated lesions ($\Delta\beta = 23\%$; false discovery rate = 0.01). Epigenome-wide methylation profiling has revealed numerous differentially methylated CpGs in normal mucosa from SPS patients. Significant hypermethylation of *HLA-F* is a novel biomarker candidate for SPS. *(J Mol Diagn 2022, 24: 674—686; https://doi.org/10.1016/j.jmoldx.2022.03.010)*

Supported by the Instituto de Salud Carlos III through the projects PI16/00766 and PI19/01867 (cofunded by European Regional Development Fund/European Social Fund; A way to make Europe/Investing in your future); supported by the Spanish Association of Gastroenterology (Asociación Española de Gastroenterología); in part cofunded by the Hospital Clínic's Premi Fi de Residència (G.J.) and by the public Catalan Health Department, Generalitat de Catalunya, Departament de Salut (PERIS 2019-2021), resolution SLT/3104/2018 (G.J.); and supported by the National Cancer Institute, NIH, grants CA72851, CA184792, CA187956, and CA202797 (A.G.).

G.J. and E.H.-I. contributed equally to this work.

Disclosures: F.B. has received an honorarium for consultancy from Sysmex, is Editor of the journal *Gastroenterología y Hepatología* (published by Elsevier), and has received speaker's fees from Norgine. The other authors declare no competing interests.

https://doi.org/10.1016/j.jmoldx.2022.03.010

Serrated polyposis syndrome (SPS) is characterized by the presence of multiple and/or large serrated polyps along the colorectum. According to the 2019 updated World Health Organization definition, at least one of the following two criteria need to be fulfilled to establish the diagnosis of SPS: at least five serrated polyps proximal to the rectum, of which at least two are $\geq$10 mm; and >20 serrated polyps of any size along the colon, of which at least five are located proximal to the rectum.[1] Serrated polyposis syndrome is currently perceived as a highly relevant disease because of three reasons: first, with increasingly implemented colorectal cancer (CRC) screening programs, the prevalence of SPS seems to be much higher than previously assumed (approximately 1 in 200 screening colonoscopies in fecal occult blood test screening programs)[2]; second, given their morphologic characteristics (pale, small, flat, and often covered by mucus), serrated lesions are often missed and the prevalence of SPS is likely even higher than the actual reported[3]; and, third, SPS has been associated with a higher risk for developing CRC and therefore surveillance programs need to be implemented.[4–6]

In addition to the long-known adenoma-carcinoma sequence, around 10% to 15% of all sporadic CRCs are believed to arise from the serrated pathway, with the serrated lesion as their precursor.[7,8] However, the enormous phenotypic, genomic, and epigenomic heterogeneity of serrated lesions is posing a challenge in sorting out the underlying molecular mechanisms, which are still widely unknown. In this sense, the serrated pathway can arise from hyperplastic polyps (HPs), sessile serrated lesions (SSLs), and traditional serrated adenomas, and all show different morphologic characteristics and molecular features. In addition, some SSLs might progress to serrated adenocarcinoma, whereas others develop as conventional CRC with histologic and molecular features of microsatellite instability high cancers.[9] Nonetheless, one common histologic feature of all the subtypes is the saw-tooth shape of the crypt base, which is believed to arise from altered apoptotic pathways.[10,11] Serrated polyposis syndrome has been associated with hypermethylation of multiple gene promoters. In a study published in 2006, Minoo et al[12] analyzed the DNA methylation at promoters of 14 markers and found a higher methylation level in patients with SPS compared with those with serrated lesions but without fulfilling criteria for SPS. More important, these differences were even more evident when normal mucosa was analyzed.[12] More recent studies have shown that, compared with conventional adenomas, SSLs show more hypermethylation, particularly in CpG islands and shores.[13] In addition, an important fraction of serrated CRCs present the so-called CpG island methylator phenotype (CIMP), and this is associated to various molecular features and risk factors, including *BRAF* mutation, microsatellite instability, proximal tumor site, female sex, older age, and smoking.[14] Another frequent finding of sporadic CRCs, overlapping with the serrated pathway, is the presence of microsatellite instability secondary to *MLH1* promoter hypermethylation.[12] A third molecular mechanism hypothesized to initiate the serrated pathway is based on activating mutations in the mitogen-activated protein kinase pathway. Particularly, *BRAF* mutations have been associated with a high level of promoter methylation and *MLH1* silencing.[15,16]

The observation that patients with a history of colorectal polyps or cancer are more likely to develop metachronous lesions than those without such previous lesions has led to the field cancerization (or field defect) hypothesis.[17,18] Thus far, some genetic and epigenetic alterations have been identified that arise in the colonic epithelium in a patchy way (fields) and predispose to the formation of polyps and eventually cancer, yet the mucosa appears macroscopically and microscopically normal.[19] This has become particularly evident for proximal serrated lesions where aberrant DNA methylation of a specific gene panel (CIMP panel) detected in normal mucosa was associated with the presence of advanced proximal serrated lesions.[20] From a clinical viewpoint, it has been proposed that these aberrations in normal mucosa could be leveraged as a tool to identify patients early at risk for harboring or developing lesions.[12] However, the clinical implications of this hypothesis remain largely unexplored.

Intense colonoscopy surveillance for SPS is costly and uncomfortable for patients. Besides, recent studies suggest that the risk of developing cancer under surveillance is low and that lower-risk patients could benefit from relaxation of the intervals.[5] To optimize patient management, it is clear that we first need to better understand the molecular fundamentals of the serrated pathway to then dispose of molecular biomarkers for a better cancer risk prediction.

This study describes the methylation profile of normal mucosa in a large cohort of SPS and identified biomarker candidates for SPS. We also aimed to verify the field defect hypothesis by comparing the methylation profiles found in normal mucosa and in serrated polyp tissue.[18]

Materials and Methods

Study Design

This study consisted of four phases (Figure 1). In the exploratory phase, a genome-wide methylation analysis was performed using the 850K Epic BeadChip Array (Infinium) in fresh-frozen tissue samples of normal colon mucosa from 50 patients diagnosed with SPS and 19 individuals without colorectal neoplasia. Because aberrant methylation has been shown to differ along the colorectum,[21] samples from the proximal and distal colon were obtained. Proximal colon was defined as proximal to the splenic flexure. The gene expression was then analyzed in the same cohort and samples and the expression levels were correlated with the methylation status of the corresponding differentially methylated regions (DMRs). In the next step, the results in formalin-fixed, paraffin-embedded (FFPE) tissue samples of

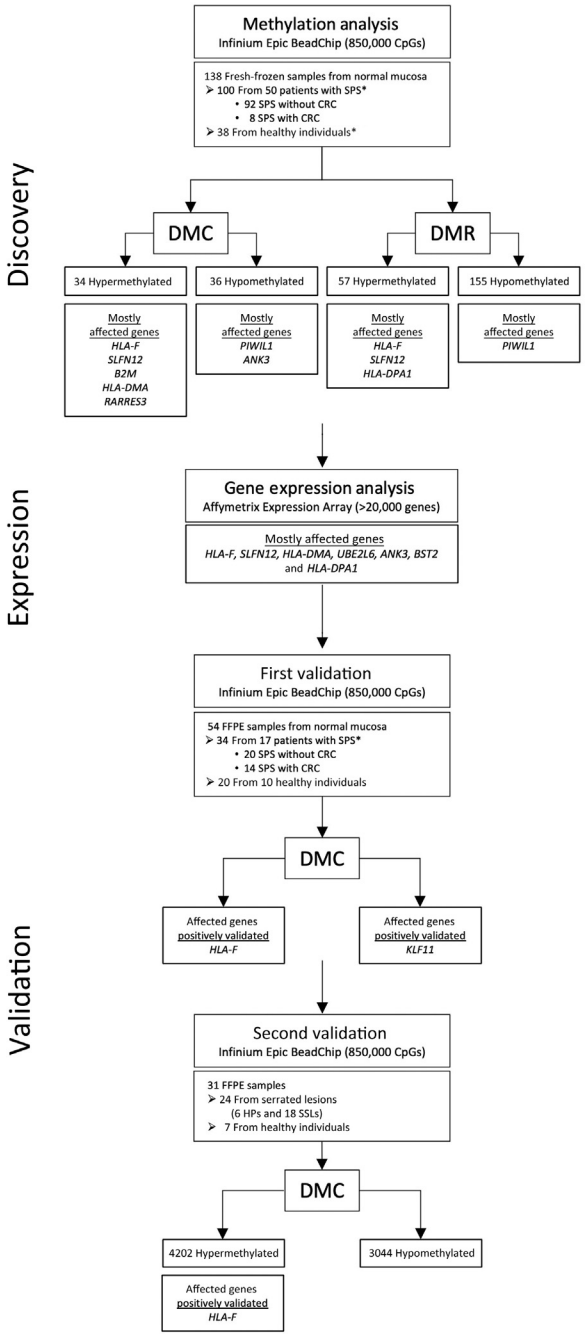

Figure 1 Study flowchart. *From each patient, one sample from the proximal colon and one sample from the distal colon were obtained. CRC, colorectal cancer; DMC, differentially methylated CpG; DMR, differentially methylated region; FFPE, formalin fixed, paraffin embedded; HP, hyperplastic polyp; SSL, sessile serrated lesion; SPS, serrated polyposis syndrome.

the normal mucosa of an independent cohort of 17 SPS patients and 10 individuals without colorectal neoplasia were validated. As a further validation step, genome-wide methylation analysis was performed using the same technique to interrogate differentially methylated CpGs directly in FFPE tissue of serrated lesions derived from another independent cohort: 6 HPs and 18 SSLs.

Study Population

Patients with SPS for the exploratory phase were prospectively recruited between 2016 and 2018 at the High-Risk CRC Clinic from the Hospital Clínic of Barcelona (Barcelona, Spain). For the validation cohort, patients were recruited retrospectively from the same clinic attended between 2004 and 2016.

Patients included in this study fulfilled criterion I and/or III, according to the 2010 World Health Organization criteria: criterion I, five or more serrated polyps proximal to the sigmoid and at least two of them measure $\geq$10 mm; and criterion III, $\geq$20 serrated polyps of any size at any localization.

Healthy controls with a normal colonoscopy (ie, without any adenoma, serrated lesion, or any other lesion) in the exploratory and validation phase derived from the CRC screening programs at the General Hospital of Alicante and the Hospital Clinic of Barcelona, respectively. In both cases, they were matched for age and sex with the study cohorts. Briefly, in these average risk, population-based screening programs, asymptomatic residents, aged 50 to 69 years, are identified by the community health registry and invited for CRC screening based on fecal immunochemical testing.[22] In those with a positive result (>155 ng Hb/mL; OC-SENSOR; Eiken Chemical Co., Taito-ku, Tokyo, Japan), a colonoscopy is performed. In this program, patients with a history of CRC, adenoma, inflammatory bowel disease, family history of hereditary or familial CRC, severe comorbidity, or previous colectomy are excluded, because these patients are considered high risk and may be under different programs of surveillance or screening. Those patients with a normal colonoscopy (ie, without any pathology) were eligible to serve as healthy controls for the present study, and biopsies from the proximal and distal colon were taken.

The study was conducted according to the guidelines of the Declaration of Helsinki. Informed consent was retrieved from all patients, and the study was approved by our local ethics committee by February 29, 2016 (registered HCB/2016/0099).

Tissue Samples

Fresh-Frozen Samples (Exploratory Cohort)

Fresh-frozen normal mucosa tissue samples (from the proximal and distal colon) from patients with SPS were obtained during the surveillance colonoscopies.[1,23] After the biopsy, samples were stored in RNAlater solution (reference AM7021; Invitrogen, Waltham, MA) at −80°C in the freezer at the laboratories of Institut d'Investigacions Biomèdiques August Pi i Sunyer (Barcelona, Spain).

FFPE Samples (Validation Cohort)

FFPE tissue samples of normal mucosa from patients who had previously received partial or total colectomy because

of SPS with ($N = 7$) or without ($N = 10$) CRC were obtained. Samples were collected from the proximal and distal colon. An expert pathologist (M.C.) confirmed histologically that the sample only contained normal mucosa and not tumor or serrated lesions. The epithelial compartment comprised about 80% to 90% of each sample and therefore microdissection was not performed.

Tissue Samples from Serrated Lesions

Serrated polyp samples that were enrolled as a second validation step were obtained by endoscopic mucosal resection between 2014 and 2018. Each serrated polyp was classified as HP and SSL to the World Health Organization classification.

DNA and RNA Extraction

Fresh-frozen samples were disaggregated by using UFO beads (Stainless Steel UFO Beads 3.5 mm RNAse free; reference F7SSUFO35-RNA; Cultek, Madrid, Spain) and the TissueLyser LT (reference 85600; Qiagen, Hilden, Germany). Genomic DNA from the tissue samples was extracted using the PureLink Genomic DNA MiniKit following the manufacturer's instructions (reference K1820-02; Invitrogen, ThermoFisher Scientific, Waltham, MA).

Table 1 Study Population

Characteristic	SPS group	Healthy controls
Discovery Phase		
Total, *n*	50	19
Age, mean (SD), years	59 (7.5)	62 (4.9)
Sex: female, *n* (%)	19 (38)	6 (32)
Tobacco use, *n* (%)		
Smoker	26 (52)	4 (21)
Nonsmoker	13 (26)	3 (16)
Unknown	11 (22)	12 (63)
2010 WHO subtype, *N* (%)		NA
I	15 (30)	
III	15 (30)	
I + III	20 (40)	
CRC, *n* (%)	4 (8)	NA
Validation Phase		
Total, *N*	17	10
Age, mean (SD), years	60 (5.0)	62 (6.4)
Sex: female, *n* (%)	9 (53)	6 (60)
Tobacco use, *n* (%)		
Smoker	10 (59)	7 (70)
Nonsmoker	3 (18)	3 (30)
Unknown	4 (23)	0 (0)
2010 WHO subtype, *n* (%)		NA
I	4 (24)	
III	6 (35)	
I + III	7 (41)	
CRC, *n* (%)	7 (41)	NA

CRC, colorectal cancer; NA, not applicable; SPS, serrated polyposis syndrome; WHO, World Health Organization.

The double-stranded DNA concentration was measured by a fluorometric method (Qubit 3.0; reference Q33216; Invitrogen). For gene expression analysis, RNA was extracted using the Rneasy miniKit following the manufacturer's instructions (reference 74104; Qiagen). Quality and quantity of RNA samples were measured on an automated electrophoresis TapeStation System Device (RNA ScreenTape Analysis; Agilent Technologies, Santa Clara, CA). The extracted DNA and RNA were then stored at −20°C and −80°C, respectively, which allowed adequate preservation of the quality of DNA and RNA for subsequent analysis of methylation and gene expression, respectively.

Human Methylation 850K Epic BeadChip Array

Global methylome analysis was performed by using an 850K Epic BeadChip Array (reference WG-317-1003; Illumina, San Diego, CA). This kit targets >850,000 CpG sites on a single-nucleotide level and covers CpGs located in CpG islands and outside of islands (ie, in shores and opensea areas). A CpG island is defined as a region of 500 to 2000 nucleotides, of which >55% are CpGs. The shores extend around 2000 bp upstream and downstream from the islands, whereas opensea represents areas with CpGs not related to CpG islands and that have a low CpG content. On the other hand, the 850K Epic BeadChip Array (Infinium) covers CpGs located in promoter regions but also in gene bodies and intergenic regions. Promoter regions of miRNAs were also covered.

In the case of the FFPE samples, the Infinium HD FFPE DNA Restore Kit (reference WG-321-1002; Illumina) was used previously to allow DNA recovery for the subsequent analysis using the Infinium platform.[24] Briefly, 1 μg of DNA was treated with sodium bisulfite (EZ DNA Methylation-Gold kit; reference D5005; Zymo Research, Tustin, CA). Subsequently, an isothermal amplification was performed at 37°C, followed by enzymatic fragmentation, purification, and hybridization (all according to the manufacturer's instructions).

The sets of methylation data were extracted using the GenomeStudio software v2011.1 (Illumina). Methylation values for individual CpG sites were obtained as β-values, calculated as the ratio of the methylated signal intensity/the sum of both methylated and unmethylated signals after background subtraction. The β-values were reported as a DNA methylation score, ranging from 0 (completely unmethylated) to 1 (completely methylated). To correct for the bias introduced by different bead types in the methylation array, a β-mixture quantile normalization method, described by Teschendorff et al[25] (2013), and which is included in the R package Chip Analysis Methylation Pipeline (ChAMP) for Infinium Illumina EPIC chips was used.[26] The batch effect was corrected by using the open-source Bioconductor package ChAMP version 2.8.9 with default parameters.[27] This R package also generates the gene set enrichment analysis and DMR analysis (ie, differentially methylated regions). A differentially methylated

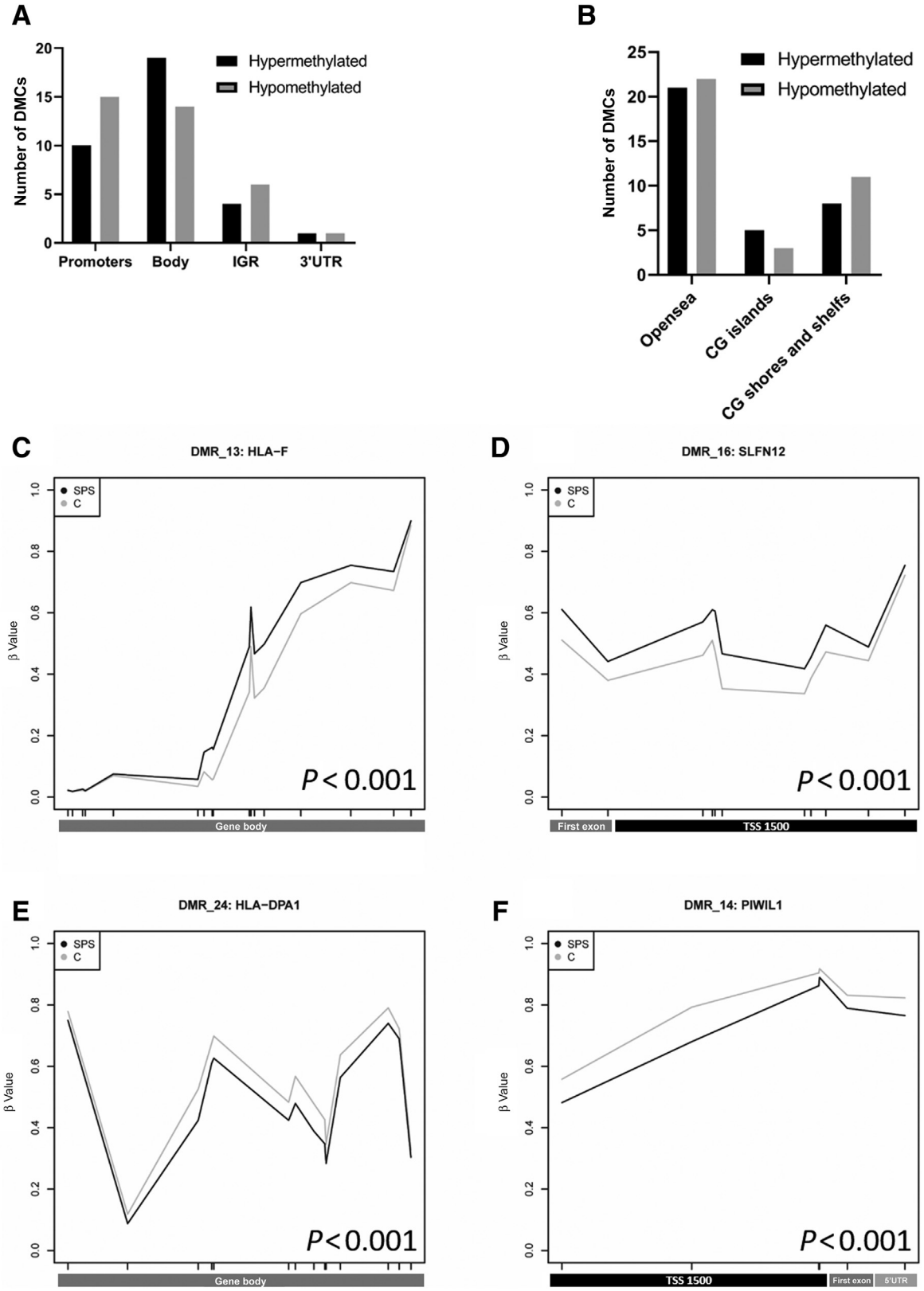

Figure 2 Distribution of differentially hypermethylated (black) and hypomethylated (gray) CpG sites, according to location relative to genes (**A**) and to CG density (**B**). Differentially methylated regions of *HLA-F* (**C**), *SLFN12* (**D**), *HLA-DPA1* (**E**), and *PIWIL1* (**F**). Both lines represent the mean β-values (*y* axis) across the gene region (*x* axis). **Black line** indicates patients with serrated polyposis syndrome (SPS); and **gray line**, healthy controls (Cs). Each tic on the *x* axis represents a specific differentially methylated CpG (DMC) and shows its location relative to the gene region. Bars below the *x* axis: black indicates transcription start site (TSS) 1500 (ie, promoter region); dark gray, gene body or first exon; and light gray, 5′ untranslated region (UTR). IGR, intergenic region.

region is a set of multiple CpGs, usually adjacent to each other, which display a predicted strong functional and regional correlation alongside the genome and possibly form a functional unit for the transcriptional regulation.[28] To detect DMRs between two populations on normalized β-values, the Bioconductor package bump hunter was used with default parameters,[29] included in the ChAMP. DMR function was assessed, to estimate regions for which a genomic profile deviates from its baseline value.

Differentially methylated cytosines and regions between groups (SPS and healthy) were assessed by linear models,[30] including clinical covariates: age, sex, colon location (proximal and distal), and tobacco use after a logit transforming of β-values. Because differences in normal mucosa were predicted to be subtle, a difference in the relative methylation level (Δβ value) of >0.1 (ie, an absolute 10% increase or decrease) was defined as clinically relevant and an adjusted *P* value (Benjamini-Hochberg method) of <0.05 as statistically significant (adjusted for sex, age, tobacco use, and location). In the case of polyp tissue, a Δβ value of >0.2 (ie, an absolute 20% increase or decrease) was defined as clinically relevant and an adjusted *P* value of <0.05 as statistically significant.

Gene Expression Analysis

A next-generation, gene-level, expression-profiling tool was used according the manufacturer's protocol, to analyze the expression levels of >20,000 genes in a transcriptome-wide manner (Human Clariom S Assay; reference 902927; ThermoFisher Scientific). The raw gene expression was processed using bioconductor tools.[31] Briefly, the entrez-based probe definition,[32] normalization using rma, and differential expressed ranking using moderated *t*-statistics were used.[30] Correlation between genes and differentially methylated regions was computed using Spearman correlation. A fold change of at least ±1.1 (ie, a 10% increase or decrease in expression level) with a *P* value of <0.05 was considered as relevant and statistically significant.

Results

Characteristics of the Study Cohorts

Clinical characteristics of the study cohorts are summarized in Table 1. Mean age in the discovery phase was 59 and 62 years for patients with SPS and healthy controls, respectively. Of the 50 SPS cases, four patients had CRC (8%) in the discovery phase. In the validation cohort, mean age was 60 and 62 years for patients with SPS and healthy individuals, respectively. There were seven cases of CRC in the SPS group (41%).

Discovery of Differentially Methylated CpGs in Fresh-Frozen Tissue

The aim was to identify differentially methylated CpGs (DMCs) as potential biomarkers for patients with SPS. Following the field defect hypothesis, we sought to identify DMCs in normal mucosa between patients with SPS and healthy controls. After adjusting for age, sex, colon location (proximal and distal), and tobacco use, 34 differentially hypermethylated and 36 differentially hypomethylated CpGs were found (Supplemental Table S1). Both events, hypermethylation and hypomethylation, occurred predominantly in promoter regions and gene bodies and not in intergenic regions (Figure 2A). Opensea areas (ie, CpGs unrelated to CpG islands) were almost twice as frequently affected as CpG islands or shores (Figure 2B). Those differentially hypermethylated and hypomethylated CpGs not related to intergenic regions corresponded to 18 and 27 genes, respectively (Supplemental Table S1). For most genes, only one CpG site was affected; however, some showed a differential methylation level at multiple CpG sites. The most hypermethylated CpG regions (ie, with more than one CpG site affected) were *HLA-F*, *SLFN12*, *B2M*, *HLA-DMA*, and *RARRES3*, and the most hypomethylated genes were *PIWIL1* and *ANK3* (Supplemental Table S1).

Because SPS subtypes are phenotypically different, their methylation profiles were next studied separately (Supplemental Figure S1). No significant differences were observed between World Health Organization 2010 subtype I ($N = 15$) and subtype III ($N = 15$) (Supplemental Table S2 and Supplemental Figure S1). When compared with normal mucosa of healthy individuals, subtype III did not show any relevant aberration in its methylation profile (Supplemental Table S3), whereas subtype I showed eight DMCs (all hypomethylated), seven of which corresponded to six genes and one to an intergenic region (Supplemental Table S4).

The methylation profiles were also compared between SPS patients with history of CRC ($N = 4$) and those without CRC ($N = 46$), but no substantial differences were observed. Only four CpGs were statistically significant after adjustment by Benjamini-Hochberg method (cg11699261, cg23979401, cg20106822, and cg12842231). All four CpGs were located in opensea areas and in the gene body of their corresponding gene: *PBLD*, *LOC728723*, *MYOM2*, and *KIAA1671* (Supplemental Table S5).

Discovery of Differentially Methylated Regions in Fresh-Frozen Tissue

Differential methylation of a single CpG site is highly relevant for biomarker discovery. However, because previous studies have found CpGs strongly correlated across the genome in a functional and regional manner, DMRs were studied next.[28,33] DMRs are genomic regions with multiple adjacent CpG sites that are differentially methylated and possibly form a functional unit involved in transcriptional regulation. Fifty-seven differentially hypermethylated and 155 hypomethylated regions ($P < 0.05$) were retrieved between patients with SPS and healthy individuals (Supplemental Table S6). Four of these DMR-defined genes

Table 2 Differentially Methylated CpGs with Best Discriminatory Performance and Good Correlation with GE

Gene	CHR	CpG	Gene location	CpG location	ME Direction
HLA-F	6	cg12588917	Body	Island	Hyper
		cg24351901	Body	Island	Hyper
		cg15331332	Body	Shore	Hyper
		cg23892836	Body	Island	Hyper
		cg07016276	Body	Island	Hyper
		cg00504902	Body	Shore	Hyper
SLFN12	17	cg19566405	TSS1500	Opensea	Hyper
		cg05174890	TSS1500	Opensea	Hyper
		cg11346248	TSS1500	Opensea	Hyper
		cg03251655	First exon	Opensea	Hyper
		cg21697381	TSS1500	Opensea	Hyper
HLA-DMA	6	cg02806715	Body	Opensea	Hyper
		cg24129356	First exon	Opensea	Hyper
UBE2L6	11	cg27429749	Body	Shore	Hyper
ANK3	10	cg14274656	First exon	Opensea	Hypo
		cg16134349	First exon	Opensea	Hypo
BST2	19	cg11558551	TSS200	Opensea	Hyper
HLA-DPA1	6	cg12858166	3′UTR	Opensea	Hyper

(*table continues*)

CHR, chromosome; GE, gene expression; Hyper, hypermethylation; Hypo, hypomethylation; ME, gene methylation; TSS, transcription start site; UTR, untranslated region.

overlapped with our findings of the single CpG analysis described above: *HLA-F*, *SLFN12*, and *HLA-DPA1* (all three hypermethylated) and *PIWIL1* (hypomethylated) (Figure 2, C–F).

Colorectal Location Impacts Substantially on the Methylation Profile

Differential aberrant methylation has been described in the different parts of the colorectum.[34] Therefore, from each patient with SPS and healthy individual a pair of one proximal and one distal biopsy specimen were included. When stratified by location, 339 DMCs were retrieved between SPS and healthy individuals if only the proximal location was analyzed (Supplemental Table S7) and 343 DMCs if only the distal location was analyzed (Supplemental Table S8). Interestingly, an important predominance of hypomethylation events in both locations (325 of 339 proximally and 330 of 343 distally) as well as a predominance of opensea DMCs (209 of 339 proximally and 204 of 343 distally) was found. To evaluate the stability of the methylome, the profile of the healthy individuals was analyzed by age group and no significant correlation was found between age and global methylation status for the proximal or the distal location [$R = -0.12$ ($P = 0.64$) versus $R = 0.38$ ($P = 0.11$), respectively] (Supplemental Figure S2, A and B). Regarding sex, a minimum tendency of higher methylation was found for female patients, but this was only significant for the distal location ($P = 0.021$) (Supplemental Figure S2, C and D).

Correlation with Gene Expression

To validate the functional impact of aberrant methylation observed in normal mucosa, the correlation of the methylation level of the differentially methylated CpGs was further studied with the expression level of their corresponding gene in the same fresh-frozen samples. Fifty genes showed a strong correlation ($r > 0.7$; $P < 0.05$), and 1934 genes showed a moderate correlation ($0.3 < r < 0.7$; $P < 0.05$) (Supplemental Table S9). The corresponding CpGs of seven of the genes with a moderate correlation ($r > 0.3$) showed highly significant differences in their methylation levels ($\Delta\beta > 0.1$; $P < 0.0001$) and significant fold changes in their expression levels (fold change > 1.1; $P < 0.05$): *HLA-F*, *SLFN12*, *HLA-DMA*, *UBE2L6*, *ANK3*, *BST2*, and *HLA-DPA1* (Table 2 and Figure 3).

Independent Validation in FFPE Samples of Normal Mucosa of Patients with SPS

To validate the results, FFPE samples of normal-appearing mucosa from the proximal and distal colon of an independent cohort of 17 SPS patients and 10 healthy individuals were analyzed (Supplemental Table S10). When analyzed by gene, two genes could be positively validated: *HLA-F,* which showed a significant hypermethylation in both fresh-frozen and paraffin-embedded specimens; and *KLF11*, which showed a significant hypomethylation in both specimens (Table 3 and Supplemental Tables S1 and S10). Of note, *HLA-F* was one of the genes that showed a moderate correlation between its methylation status and gene

Table 2 (*continued*)

ME		GE			Correlation of ME-GE	
Δβ Methylation	*P* value	Direction	Fold change	*P* value	*r*	*P* value
0.145	1.2×10^{-11}	Down	−1.246	0.001	−0.631	0
0.144	2.4×10^{-9}					
0.142	6.7×10^{-9}					
0.129	3.5×10^{-9}					
0.101	4.6×10^{-6}					
0.102	2.2×10^{-6}					
0.130	2.2×10^{-8}	Down	−1.223	0.000	−0.583	0
0.113	7.4×10^{-7}					
0.110	6.8×10^{-8}					
0.100	1.5×10^{-7}					
0.100	7.5×10^{-8}					
0.126	1.0×10^{-11}	Down	−1.276	0.000	−0.578	0
0.121	5.3×10^{-11}					
0.117	2.2×10^{-6}	Down	−1.193	0.038	−0.521	7.3×10^{-11}
−0.117	3.0×10^{-6}	Down	−1.140	0.033	−0.409	1.0×10^{-6}
−0.104	5.0×10^{-8}					
0.102	5.0×10^{-8}	Down	−1.361	0.000	−0.380	6.0×10^{-6}
0.104	1.1×10^{-2}	Down	−1.198	0.003	−0.315	2.0×10^{-4}

expression levels ($r = -0.631$) in the exploratory phase of the study.

Similar to the analysis in fresh-frozen samples, in FFPE tissue, no statistically significant aberrant methylation profiles were found between patients with CRC ($N = 7$) and those without ($N = 10$). Of 1208 CpGs that showed >10% increase or decrease in their methylation level, none reached statistical significance after adjustment (Supplemental Table S11).

Validation in FFPE Samples of Polyp Tissue

We next hypothesized that the changes detected in normal mucosa (field defect) predispose for the development of serrated polyps and thus should be present in preneoplastic lesions. To confirm this hypothesis, the differential methylation pattern of an independent cohort of 24 serrated lesions (6 HPs and 18 SSLs) were analyzed and compared with 7 healthy controls. Because pathologic tissue was predicted to harbor more epigenetic changes compared with normal mucosa, more stringent parameters were predefined for the detection of differences in methylation levels and 27,062 significantly hypermethylated and 9677 significantly hypomethylated CpG sites were identified. When looking at DMCs in promoter regions, almost 10 times more hypermethylated DMCs (13,999 CpGs) than hypomethylated DMCs (1418 CpGs) were detected. Hypermethylated DMCs not related to intergenic regions corresponded to 4203 genes, and hypomethylated CpGs corresponded to 3044 genes (Supplemental Table S12). Furthermore, nine shared genes that harbored DMCs in both cohorts were identified, of which three were hypermethylated (*HLA-F*, *CDC42SE2*, and *RBMS1*) and six were differentially hypomethylated (*MUC21*, *ANK3*, *SNX29*, *TRERF1*, *KLF12*, and *AGPAT4*).

In summary, *HLA-F* was found to be differentially hypermethylated in fresh-frozen samples of normal mucosa of SPS patients, correlating well with gene expression, and differentially hypermethylated in FFPE samples of an independent cohort as well as in FFPE samples of serrated lesions (Figure 4).

Gene Set Enrichment Analysis

To better understand the possible functional implications of the genes related to the DMCs found herein, a gene set enrichment analysis was performed on the list of DMCs found in the fresh-frozen samples. By using the Kyoto Encyclopedia of Genes and Genomes software v92.0 (*https://www.genome.jp/kegg*), a significant enrichment was identified in 24 different gene sets with an adjusted *P* value of <0.05 (Supplemental Table S13). Overall, there was an enrichment in gene sets related to DNA methylation, cancer (also colon cancer), morphogenesis (homeobox genes), embryonic stem cell identity, cell-cell adhesion, and immune response, particularly IgA production and antigen processing and presentation. Intriguingly, none of the typical genes linked to the CIMP was enriched in our data set.

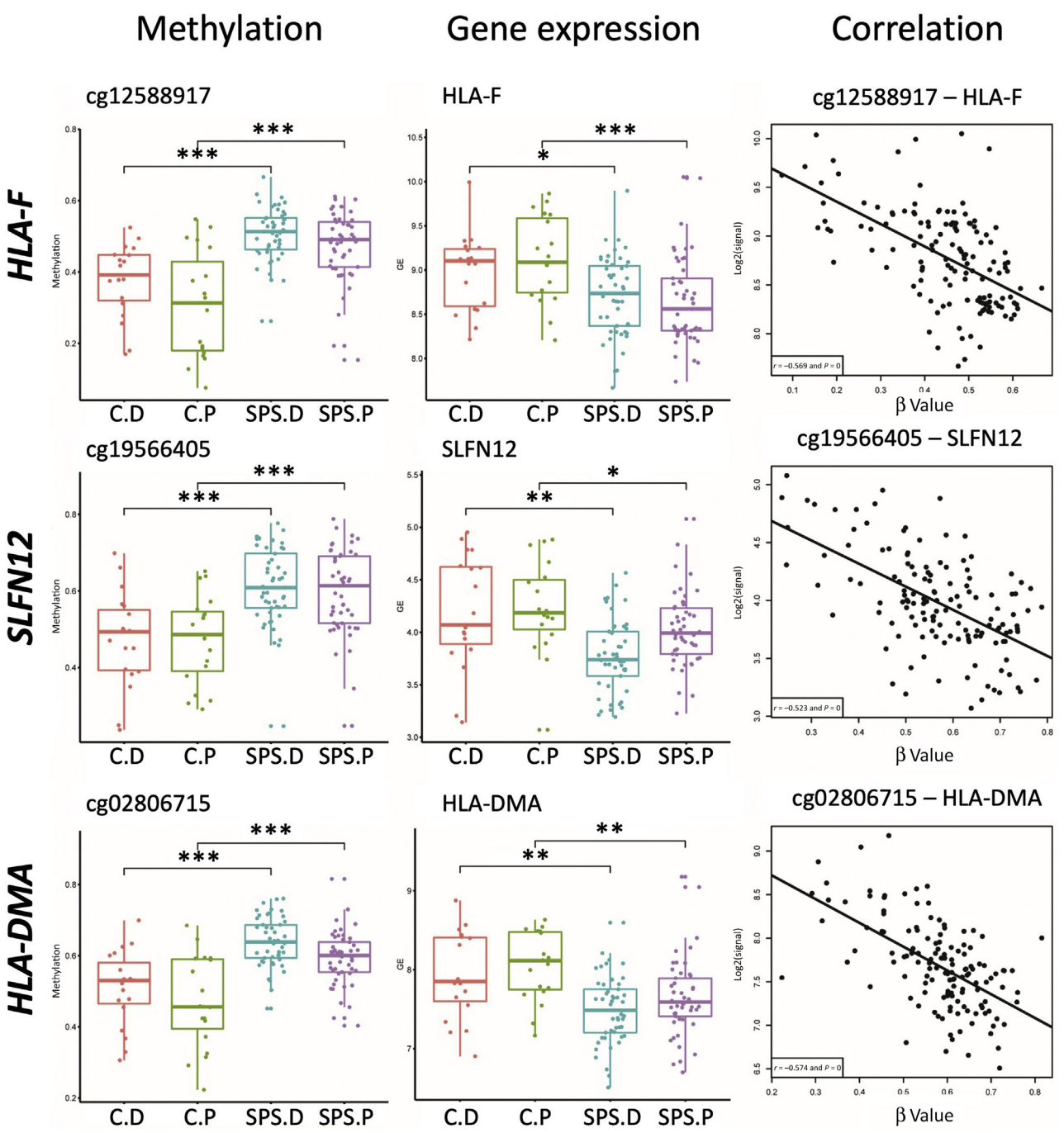

Figure 3 For the three genes (rows) with best differences in methylation levels (**left panels**) and expression levels (**middle panels**) as well as good correlation between expression and methylation (**right panels**), box-and-whisker plots and correlation curves are displayed: *HLA-F*, *SLFN12*, and *HLA-DMA*. For differences in methylation levels, one representative CpG site is shown. For both methylation and gene expression levels, differences are disclosed by group and location: distal colon of healthy control (C.D), proximal colon of healthy control (C.P), distal colon of serrated polyposis syndrome (SPS; SPS.D), and proximal colon of SPS (SPS.P). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. HLA, human leukocyte antigen.

Discussion

The pathogenesis of SPS remains elusive. In this study, for the first time, the methylation profile of >850,000 CpGs was analyzed in normal mucosa of patients with SPS to explore the field defect of cancerization in this disease. The results support the existence of a methylation field defect in normal mucosa between patients with SPS and healthy individuals. The fact that the differential methylation levels of some DMCs inversely correlated with the expression levels of their corresponding gene suggests that these phenomena have a functional impact and thus underlines

Table 3 Validated Differentially Methylated and Expressed Genes in FFPE

Gene	CHR	CpG	Methylation	Δβ	*P* value	Gene location	CpG location
HLA-F	6	cg22298860	Hyper	0.145	2.0×10^{-7}	TSS1500	Shore
		cg26751972	Hyper	0.143	4.6×10^{-6}	Body	Island
		cg11768167	Hyper	0.101	1.1×10^{-5}	TSS1500	Shore
		cg11587584	Hyper	0.101	1.6×10^{-4}	Body	Shore
KLF11	2	cg05647197	Hypo	−0.156	2.0×10^{-5}	TSS1500	Shore
		cg18424122	Hypo	−0.148	9.2×10^{-5}	TSS1500	Shore
		cg01354088	Hypo	−0.123	2.5×10^{-4}	TSS1500	Island
		cg12005760	Hypo	−0.113	1.1×10^{-4}	TSS1500	Island
		cg15123428	Hypo	−0.110	3.2×10^{-4}	Body	Shore
		cg11249298	Hypo	−0.107	1.8×10^{-8}	Body	Shore

CHR, chromosome; FFPE, formalin fixed, paraffin embedded; Hyper, hypermethylation; Hypo, hypomethylation; TSS, transcription start site.

their relevance. Finally, the hypermethylation of the gene body of *HLA-F* in formalin-fixed tissue was validated in an independent cohort and in serrated polyp tissue, highlighting it as a promising biomarker candidate for the field defect in SPS.

In a former study by Minoo et al,[12] DNA hypermethylation of a limited panel of the so-called CIMP markers was found to be more extensive in serrated polyps from patients with SPS compared with patients without criteria for SPS, although the number of patients ($N = 3$ in the SPS group, and $N = 9$ in non-SPS group) was limited. In the same study, this difference was even more apparent in normal mucosa of the proximal colon. Our results are in line with that preliminary observation using a high-throughput analysis of methylation. However, notably, no significant differences were found in any of the previously described CIMP markers in normal colonic mucosa. Instead, in serrated polyp tissue, intense promoter hypermethylation was found in most of the previously described CIMP markers (ie, *NEUROG1*, *RUNX3*, *CACNA1G*, *MLH1*, *CDKN2A*, *RASSF2*, and *MGMT*), suggesting that they may not be present at early disease stages but rather in developed serrated polyps.[35,36] Another interesting finding of our results is that, apart from CpG islands in promoters, aberrant CpGs in opensea areas and CpGs located in gene bodies represent a meaningful proportion of the differential methylation observed in normal mucosa. Nonetheless, the technology used in our study still captures only 3% to 4% of around 28 million CpG sites in the human DNA, and higher yields are expected with the advent of even more advanced technologies in the near future.

In this study, we tested the hypothesis that the methylation field defect in normal mucosa could potentially be used to identify patients with SPS at higher risk for CRC. However, no significant differences were detected in the aberrant methylation between patients with and without CRC in none of the two independent cohorts analyzed. Because the absolute numbers of cases with CRC were rather low in both cohorts (4 of 50 and 7 of 17, respectively) and because of the underlying heterogeneity of the disease, increasing the number of CRC cases may lead to significant results in larger cohorts. However, it is also possible that those alterations necessary for polyp formation and cancer progression are only measurable in the developed lesions

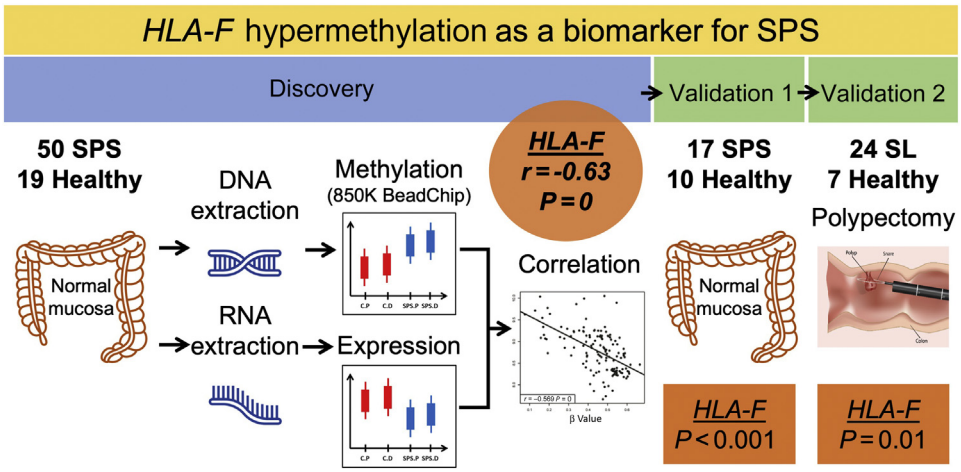

Figure 4 Summary of the design of the study and highlighting hypermethylation of *HLA-F* as the most promising biomarker candidate for serrated polyposis syndrome (SPS). SL, serrated lesions. Icons/images are licensed from *thenounproject.com* or iStock by Getty Images.

and not in the normal mucosa. This would explain the observation that a substantially higher number of DMCs were found in direct polyp tissue compared with normal-appearing mucosa.

Hypermethylation of the gene body of *HLA-F* has emerged as the most consistent biomarker candidate for SPS because it showed the highest number of DMCs, it was validated in an independent cohort of patients, and it was validated in serrated polyp tissues. In addition, *HLA-F* was significantly underexpressed in the normal mucosa, with an inverse correlation with the methylation status. In the literature, the function of *HLA-F* is still not fully elucidated, but it is clear that it participates in human peptide presentation to natural killer cell receptors and regulates immune response.[37] Furthermore, the expression of *HLA-F* and other human leukocyte antigen (HLA) molecules has been linked to several cancers of the digestive system in the literature,[38–41] but the association of its methylation status with a precancerous condition like SPS is truly a novel finding of our study. García-Solano et al,[42] in a study comparing the methylation profile of serrated adenocarcinomas and tumors with sporadic microsatellite instability, found that hypermethylation of *HLA-DOA* was associated with tumors with sporadic microsatellite instability, with an inverse correlation in the expression levels. Interestingly, *HLA-DOA* was also found to be differentially methylated in serrated polyp tissues (Supplemental Table S12) but not in the normal mucosa of patients with SPS, indicating that it may be a later event in polyp formation. Besides from *HLA-F*, other HLA molecules were found to be differentially methylated, such as HLA-DMA and HLA-DPA1 when looking at single DMCs as well as HLA-E, HLA-J, and HLA-DPA1 when looking at DMRs (Supplemental Table S6). In some cases, their higher methylation status correlated with lower expression levels. HLA class I molecules seem to play an important role for immune surveillance of cancer cells, and our study generates the hypothesis of their involvement in cancer initiation and progression in SPS.[43]

There are some limitations in this study. First, the results were not technically validated with a different technique, such as pyrosequencing.[42] However, a clinical validation was performed in an independent cohort of patients using the same method, which has been extensively demonstrated to be robust. Second, even though so far we have analyzed the largest cohort of patients with SPS for their methylation profile in normal mucosa, the number of cases was not large enough to perform an adequate subgroup analysis comparing the World Health Organization I and II criteria, or patients with and without CRC. Third, with the comparative and cross-sectional design, it is impossible to perform causal inference between methylation status and expression levels, and the significance of the identified genes and SPS carcinogenesis. There is abundant evidence for the relation between gene promoter hypermethylation and down-regulation of gene expression.[44] However, many other processes influence gene expression, such as histone modifications, miRNAs, and long noncoding RNAs, which all interplay in a complex regulatory network.[45–47] Moreover, the effects of hypermethylation or hypomethylation of intergenic regions on gene expression are more elusive, and it is likely that most of the epigenetic alterations observed are passenger events rather than causative ones.[48] Finally, this study constitutes a proof-of-concept study that supports the existence of a certain methylation field defect in SPS that needs to be validated in further investigations.

In conclusion, in this study, we describe the existence of aberrant methylation in the normal mucosa of patients with SPS, associated with changes in the expression of the affected genes. From a clinical perspective, it would be desirable not only to properly diagnose this disease, but also to detect those patients at higher risk of developing colorectal cancer to improve surveillance programs and reduce the burden of colonoscopies. Prospective studies with serial sampling of normal mucosa, serrated polyp tissue, and serrated adenocarcinoma can provide deeper pathophysiological insights and facilitate the discovery of biomarkers for cancer risk.

Conclusions

- Epigenome-wide DNA methylation analysis (by 850K InfiniumEpic BeadChip Kit) is capable of identifying numerous DMCs and DMRs in fresh-frozen samples of normal mucosa of patients with SPS.
- Two genes with DMCs have been validated in FFPE samples of an independent cohort of SPS patients: *HLA-F* and *KLF11*.
- Seven genes showed significant changes in their expression levels, and these correlated well with the methylation status of their corresponding CpGs.
- Some validated DMCs are also present in polyp tissue alongside additional epigenetic changes: *HLA-F*, *CDC42SE2*, *RBMS1*, *MUC21*, *PRR12*, *ANK3*, *SNX29*, *TRERF1*, *KLF12*, and *AGPAT4*.
- There is an enrichment in methylation changes in genes involved in cancer pathways and immune response, but their possible role in the colorectal carcinogenesis needs to be elucidated.

Author Contributions

G.J., E.H.-I., and F.B. conceptualized the study; E.H.-I., J.M., and F.B. developed the methodology; J.J.L. and J.S. were responsible for software development and coding; G.J. and J.J.L validated the data; G.J., E.H.-I., J.J.L, J.S., and E.B. formally analyzed and synthesized the study data; E.H.-I., G.J., J.M., Y.O., E.Q., S.C., L.M., M.D., T.O., A.S., L.R., O.O., and J.L. were involved in the investigation process, performed the experiments, and/or collected the data; Y.O., E.Q., G.H., M.C., A.G., E.B., R.J., A.C., and

F.B. provided resources for the study including patient samples, instrumentation, and reagents; G.J., J.J.L., J.S., and S.C. were responsible for data curation; G.J. wrote and prepared the original draft; all authors were involved in writing and reviewing the final manuscript; E.H.-I. and G.J. visualized the results and presented them at national and international conferences; A.C., A.G., M.P., and F.B. supervised the research; E.H.-I., G.J., and F.B. were the administrators of the research project; F.B., A.G., and G.J. acquired funding.

Acknowledgment

We thank the patients for their participation.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2022.03.010*.

References

1. Rosty C, Brosens LAA, Dekker E, Nagtegaal ID: Serrated Polyposis. ed 5. Lyon, France, International Agency for Research on Cancer, World Health Organization, 2019
2. IJspeert JEG, Bevan R, Senore C, Kaminski MF, Kuipers EJ, Mroz A, Bessa X, Cassoni P, Hassan C, Repici A, Balaguer F, Rees CJ, Dekker E: Detection rate of serrated polyps and serrated polyposis syndrome in colorectal cancer screening cohorts: a European overview. Gut 2017, 66:1225−1232
3. Rivero-Sanchez L, Lopez-Ceron M, Carballal S, Moreira L, Bessa X, Serradesanferm A, Pozo A, Augé JM, Ocaña T, Sánchez A, Leoz ML, Cuatrecasas M, Grau J, Llach J, Castells A, Balaguer F, Pellisé M: Reassessment colonoscopy to diagnose serrated polyposis syndrome in a colorectal cancer screening population. Endoscopy 2017, 49:44−53
4. Boparai KS, Mathus-Vliegen EMH, Koornstra JJ, Nagengast FM, van Leerdam M, van Noesel CJM, Houben M, Cats A, van Hest LP, Fockens P, Dekker E: Increased colorectal cancer risk during follow-up in patients with hyperplastic polyposis syndrome: a multicentre cohort study. Gut 2010, 59:1094−1100
5. Bleijenberg AGGC, Ijspeert JEEG, van Herwaarden YJ, Carballal S, Pellisé M, Jung G, Bisseling TM, Nagetaal ID, Van Leerdam ME, van Lelyveld N, Bessa X, Rodríguez-Moranta F, Bastiaansen B, de Klaver W, Rivero L, Spaander MCWC, Koornstra JJ, Bujanda L, Balaguer F, Dekker E: Personalised surveillance for serrated polyposis syndrome: results from a prospective 5-year international cohort study. Gut 2020, 69:112−121
6. Bleijenberg AGC, IJspeert JEG, Hazewinkel Y, Boparai KS, Oppeneer SC, Bastiaansen BAJ, Dekker E: The long-term outcomes and natural disease course of serrated polyposis syndrome: over 10 years of prospective follow-up in a specialized center. Gastrointest Endosc 2020, 92:1098−1107.e1
7. García-Solano J, Pérez-Guillermo M, Conesa-Zamora P, Acosta-Ortega J, Trujillo-Santos J, Cerezuela-Fuentes P, Mäkinen MJ: Clinicopathologic study of 85 colorectal serrated adenocarcinomas: further insights into the full recognition of a new subset of colorectal carcinoma. Hum Pathol 2010, 41:1359−1368
8. García-Solano J, García-Solano ME, Torres-Moreno D, Carbonell P, Trujillo-Santos J, Pérez-Guillermo M, Conesa-Zamora P: Biomarkers for the identification of precursor polyps of colorectal serrated adenocarcinomas. Cell Oncol 2016, 39:243−252
9. De Palma FDE, D'Argenio V, Pol J, Kroemer G, Maiuri MC, Salvatore F: The molecular hallmarks of the serrated pathway in colorectal cancer. Cancers (Basel) 2019, 11:1017
10. Tateyama H, Li W, Takahashi E, Miura Y, Sugiura H, Eimoto T: Apoptosis index and apoptosis-related antigen expression in serrated adenoma of the colorectum. Am J Surg Pathol 2002, 26:249−256
11. O'Brien MJ, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, Amorosino M, Farraye FA: Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. Am J Surg Pathol 2006, 30:1491−1501
12. Minoo P, Baker K, Goswami R, Chong G, Foulkes WD, Ruszkiewicz AR, Barker M, Buchanan D, Young J, Jass JR: Extensive DNA methylation in normal colorectal mucosa in hyperplastic polyposis. Gut 2006, 55:1467−1474
13. Parker HR, Orjuela S, Martinho Oliveira A, Cereatti F, Sauter M, Heinrich H, Tanzi G, Weber A, Komminoth P, Vavricka S, Albanese L, Buffoli F, Robinson MD, Marra G: The proto CpG island methylator phenotype of sessile serrated adenomas/polyps. Epigenetics 2018, 13:1088−1105
14. Weisenberger DJ, Levine AJ, Long TI, Buchanan DD, Walters R, Clendenning M, Rosty C, Joshi AD, Stern MC, Le Marchand L, Lindor NM, Daftary D, Gallinger S, Selander T, Bapat B, Newcomb PA, Campbell PT, Casey G, Ahnen DJ, Baron JA, Haile RW, Hopper JL, Young JP, Laird PW, Siegmund KD; Colon Cancer Family Registry: Association of the colorectal CpG island methylator phenotype with molecular features, risk factors, and family history. Cancer Epidemiol Biomarkers Prev 2015, 24:512−519
15. Rad R, Cadiñanos J, Rad L, Varela I, Strong A, Kriegl L, Constantino-Casas F, Eser S, Hieber M, Seidler B, Price S, Fraga MF, Calvanese V, Hoffman G, Ponstingl H, Schneider G, Yusa K, Grove C, Schmid RM, Wang W, Vassiliou G, Kirchner T, McDermott U, Liu P, Saur D, Bradley A: A genetic progression model of Braf V600E-induced intestinal tumorigenesis reveals targets for therapeutic intervention. Cancer Cell 2013, 24:15−29
16. Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N, Matsubara N: Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol 2004, 22:4584−4594
17. Bernstein C, Bernstein H, Payne CM, Dvorak K, Garewal H: Field defects in progression to gastrointestinal tract cancers. Cancer Lett 2008, 260:1−10
18. Luo Y, Yu M, Grady WM: Field cancerization in the colon: a role for aberrant DNA methylation? Gastroenterol Rep 2014, 2:16−20
19. Belshaw NJ, Elliott GO, Foxall RJ, Dainty JR, Pal N, Coupe A, Garg D, Bradburn DM, Mathers JC, Johnson IT: Profiling CpG island field methylation in both morphologically normal and neoplastic human colonic mucosa. Br J Cancer 2008, 99:136−142
20. Worthley DL, Whitehall VLJ, Buttenshaw RL, Irahara N, Greco SA, Ramsnes I, Mallitt KA, Le Leu RK, Winter J, Hu Y, Ogino S, Young GP, Leggett BA: DNA methylation within the normal colorectal mucosa is associated with pathway-specific predisposition to cancer. Oncogene 2010, 29:1653−1662
21. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS: CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 2009, 58:90−96
22. Castells A, Quintero E: Programmatic screening for colorectal cancer: the COLONPREV study. Dig Dis Sci 2015, 60:672−680

23. van Leerdam ME, Roos VH, van Hooft JE, Dekker E, Jover R, Kaminski MF, Latchford A, Neumann H, Pellisé M, Saurin J-C, Tanis PJ, Wagner A, Balaguer F, Ricciardiello L: Endoscopic management of polyposis syndromes: European Society of Gastrointestinal Endoscopy (ESGE) guideline. Endoscopy 2019, 51:877−895
24. De Ruijter TC, De Hoon JP, Slaats J, De Vries B, Janssen MJ, Van Wezel T, Aarts MJ, Van Engeland M, Tjan-Heijnen VC, Van Neste L, Veeck J: Formalin-fixed, paraffin-embedded (FFPE) tissue epigenomics using Infinium HumanMethylation450 BeadChip assays. Lab Invest 2015, 95:833−842
25. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, Beck S: A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 2013, 29:189−196
26. Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, Beck S: ChAMP: 450k Chip analysis methylation pipeline. Bioinformatics 2014, 30:428−430
27. Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, Teschendorff AE: ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics 2017, 33:3982−3984
28. Rakyan VK, Down TA, Balding DJ, Beck S, Rakyan VK, Down TA, Balding DJ, Beck S: Epigenome-wide association studies for common human diseases. Nat Rev Genet 2011, 12:529−541
29. Jaffe AE, Murakami P, Lee H, Leek JT, Fallin MD, Feinberg AP, Irizarry RA: Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. Int J Epidemiol 2012, 41: 200−209
30. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK: Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015, 43:e47
31. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JYH, Zhang J: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004, 5:R80
32. Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F: Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res 2005, 33:e175
33. Vanderkraats ND, Hiken JF, Decker KF, Edwards JR: Discovering high-resolution patterns of differential DNA methylation that correlate with gene expression changes. Nucleic Acids Res 2013, 41: 6816−6827
34. Yamauchi M, Morikawa T, Kuchiba A, Imamura Y, Qian ZR, Nishihara R, Liao X, Waldron L, Hoshida Y, Huttenhower C, Chan AT, Giovannucci E, Fuchs C, Ogino S: Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. Gut 2012, 61:847−854
35. Rhee Y-Y, Kim K-J, Kang GH: CpG island methylator phenotype-high colorectal cancers and their prognostic implications and relationships with the serrated neoplasia pathway. Gut Liver 2017, 11: 38−46
36. Jia M, Gao X, Zhang Y, Hoffmeister M, Brenner H: Different definitions of CpG island methylator phenotype and outcomes of colorectal cancer: a systematic review. Clin Epigenetics 2016, 8:1−14
37. Dulberger CL, McMurtrey CP, Hölzemer A, Neu KE, Liu V, Steinbach AM, Garcia-Beltran WF, Sulak M, Jabri B, Lynch VJ, Altfeld M, Hildebrand WH, Adams EJ: Human leukocyte antigen F presents peptides and regulates immunity through interactions with NK cell receptors. Immunity 2017, 46:1018−1029.e7
38. Wu B, Yang H, Ying S, Lu H, Wang W, Lv J, Xiong H, Hu W: High HLA-F expression is a poor prognosis factor in patients with nasopharyngeal carcinoma. Anal Cell Pathol (Amst) 2018, 2018: 7691704
39. Ishigami S, Arigami T, Okumura H, Uchikado Y, Kita Y, Kurahara H, Maemura K, Kijima Y, Ishihara Y, Sasaki K, Uenosono Y, Natsugoe S: Human leukocyte antigen (HLA)-E and HLA-F expression in gastric cancer. Anticancer Res 2015, 35: 2279−2285
40. Xu Y, Han H, Zhang F, Lv S, Li Z, Fang Z: Lesion human leukocyte antigen-F expression is associated with a poor prognosis in patients with hepatocellular carcinoma. Oncol Lett 2015, 9:300−304
41. Zhang X, Lin A, Zhang JG, Bao WG, Xu DP, Ruan YY, Yan WH: Alteration of HLA-F and HLA i antigen expression in the tumor is associated with survival in patients with esophageal squamous cell carcinoma. Int J Cancer 2013, 132:82−89
42. García-Solano J, Turpin MC, Torres-Moreno D, Huertas-López F, Tuomisto A, Mäkinen MJ, Conesa A, Conesa-Zamora P: Two histologically colorectal carcinomas subsets from the serrated pathway show different methylome signatures and diagnostic biomarkers. Clin Epigenetics 2018, 10:141
43. Kochan G, Escors D, Breckpot K, Guerrero-Setas D: Role of non-classical MHC class I molecules in cancer immunosuppression. Oncoimmunology 2013, 2:e26491
44. Khamas A, Ishikawa T, Mogushi K, Iida S, Ishiguro M, Tanaka H, Uetake H, Sugihara K: Genome-wide screening for methylation-silenced genes in colorectal cancer. Int J Oncol 2012, 41:490−496
45. Cedar H, Bergman Y: Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 2009, 10: 295−304
46. Poddar S, Kesharwani D, Datta M: Interplay between the miRNome and the epigenetic machinery: implications in health and disease. J Cell Physiol 2017, 232:2938−2945
47. Jung G, Hernández-Illán E, Moreira L, Balaguer F, Goel A: Epigenetics of colorectal cancer: biomarker and therapeutic potential. Nat Rev Gastroenterol Hepatol 2020, 17:111−130
48. Kalari S, Pfeifer GP: Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 2010, 70: 277−308