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ORIGINAL ARTICLE

Basic Study

Aging related methylation influences the gene expression of key control genes in colorectal cancer and adenoma

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Abstract

AIM

To analyze colorectal carcinogenesis and age-related DNA methylation alterations of gene sequences associated with epigenetic clock CpG sites.

METHODS

In silico DNA methylation analysis of 353 epigenetic clock CpG sites published by Steve Horvath was performed using methylation array data for a set of 123 colonic tissue samples [64 colorectal cancer (CRC), 42 adenoma, 17 normal; GEO accession number: GSE48684]. Among the differentially methylated age-related genes, secreted frizzled related protein 1 (*SFRP1*) promoter methylation was further investigated in colonic tissue from 8 healthy adults, 19 normal children, 20 adenoma and 8 CRC patients using bisulfite-specific PCR followed by methylation-specific high resolution melting (MS-HRM) analysis. mRNA expression of age-related "epigenetic clock" genes was studied using Affymetrix HGU133 Plus2.0 whole transcriptome data of 153 colonic biopsy samples (49 healthy adult, 49 adenoma, 49 CRC, 6 healthy children) (GEO accession numbers: GSE37364, GSE10714, GSE4183, GSE37267). Whole promoter methylation analysis of genes showing inverse DNA methylation-gene expression data was performed on 30 colonic samples using methyl capture sequencing.

RESULTS

Fifty-seven age-related CpG sites including hypermethylated *PPP1R16B*, *SFRP1*, *SYNE1* and hypomethylated MGP, PIPOX were differentially methylated between CRC and normal tissues (P < 0.05, $\Delta\beta \geqslant$ 10%). In the adenoma *vs* normal comparison, 70 CpG sites differed significantly, including hypermethylated *DKK3*, *SDC2*, *SFRP1*, *SYNE1* and hypomethylated *CEMIP*, *SPATA18* (P < 0.05, $\Delta\beta \geqslant$ 10%). In MS-HRM analysis, the SFRP1 promoter region was significantly hypermethylated in CRC (55.0% ± 8.4 %) and adenoma tissue samples (49.9% ± 18.1%) compared to normal adult (5.2% ± 2.7%) and young (2.2% ± 0.7%) colonic tissue (P < 0.0001). DNA methylation of *SFRP1* promoter was slightly, but significantly increased in healthy adults compared to normal young samples (P < 0.02). This correlated with significantly increased *SFRP1* mRNA levels in children compared to normal adult samples (P < 0.05). In CRC tissue the mRNA expression of 117 age-related genes were changed, while in adenoma samples 102 genes showed differential expression compared with normal colonic tissue (P < 0.05, logFC > 0.5). The change of expression for several genes including *SYNE1*, *CLEC3B*, *LTBP3* and *SFRP1*, followed the same pattern in aging and carcinogenesis, though not for all genes (*e.g.*, MGP).

CONCLUSION

Several age-related DNA methylation alterations can be observed during CRC development and progression affecting the mRNA expression of certain CRC- and adenoma-related key control genes.

Key words: DNA methylation; Aging; Colorectal cancer; Adenoma; Epigenetic drift; Epigenetic clock; Secreted frizzled related protein 1

Core tip: Several age-related DNA methylation alterations could be observed during colorectal cancer (CRC) formation and progression affecting the mRNA expression of certain CRC- and adenoma-related key control genes such as hypermethylated secreted frizzled related protein 1 (*SFRP1*), spectrin repeat containing nuclear envelope protein 1 and hypomethylated cell migration-inducing protein. For the first time significantly lower *SFRP1* methylation levels were demonstrated in colonic tissue from children (under 18 years) compared to healthy adults. The main CRC-associated signal transduction pathways, such as WNT signaling and PI3K/Akt pathways are also influenced during aging.

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INTRODUCTION

DNA methylation alterations in connection with aging include epigenetic drift and epigenetic clock phenomena. Epigenetic drift is defined as the global DNA methylation changes caused by random and environmental individual-specific factors, while the epigenetic clock is defined as a group of progressive age-related epigenetic alterations at specific genomic sites which are common across individuals and occassionally across various tissue types[1,2]. The epigenetic clock concept is an approach to biological age prediction of different tissues based on the DNA methylation status of 353 CpG sites measured using the Illumina Beadchip450K methylation array platform[2].

Although age-related (A type) and cancer-related (C type) DNA methylation are often distinguished, the main age-related disease is cancer and the age of patients is one of the risk factor for carcinogenesis[3]. In human development, following a transient increase in average DNA methylation in early childhood (during the first year of life)[4,5], global hypomethylation is characteristic during aging[6,7]. Similarly global hypomethylation is observed in various types of cancers including colorectal cancer (CRC)[8]. With aging, besides global hypomethylation, local hypermethylation can occur on promoters of certain genes, including tumor suppressor gene promoters in various types of cancers, and many tumor suppressor genes were reported among the age-dependently hypermethylated genes[6]. Among others, promoter hypermethylation of *APC*[7,9-12], *CDKN2A*[7,9], *ESR1*[7,13,14], *GATA5*[15,16], *HPP1*[7,15,17], *SFRP1*[7,12,15,18-23] and *SFRP2*[7,18-21] genes was reported for colonic tissues during both aging and colorectal carcinogenesis. Although DNA methylation data from adult colonic tissue samples has been expansively published, data for children/young patients are limited.

In this study, we analyzed DNA methylation and/or gene expression changes of genes covered by the 353 epigenetic clock CpG sites[2] for patients of different ages as well as stages in the progression through to CRC in order to study the possible relationship between age-related and cancer-associated epigenetic alterations. Gene expression analysis was performed using colonic tissue samples from healthy children, healthy adults, and patients with adenomas and CRC. Among the differentially methylated/expressed age-related genes, secreted frizzled related protein 1 (*SFRP1*) promoter methylation was further analyzed in healthy, premalignant and cancerous colonic tissue samples, and to our knowledge this is the first study to also include colonic biopsy specimens from children.

MATERIALS AND METHODS

In silico DNA methylation analysis

The DNA methylation status of 353 age-related CpG sites[2] was analyzed *in silico* using methylation array data from the Illumina BeadChip450K. Analysis was performed on 123 CRC, adenoma and normal tissue samples available in the NCBI Gene Expression Database database (GEO accession number: GSE48684[24]). Differences between average methylation values of the compared diagnostic groups ($\Delta\beta$-values) and *P* values were determined for each CpG site (cg IDs). For statistical evaluation, normal distribution was checked using Kolmogorov-Smirnov test. Hence normal distribution was observed in any cases, Student's *t*-test with Benjamini and Hochberg correction was applied for paired group comparisons. Significance criteria were $P < 0.05$ in all cases.

In silico gene expression analysis

The expression of age-related "epigenetic clock" genes was analyzed using whole transcriptome data from Affymetrix HGU133 Plus2.0. Data was obtained from 153 colonic biopsy samples (49 healthy, 49 adenoma, 49 CRC and 6 healthy children) previously hybridized by our research group (GEO serial accession numbers: GSE37364[25], GSE10714[26], GSE4183[27], GSE37267[28]). Gene expression levels were compared using unpaired Student's *t*-test with Benjamini and Hochberg correction (*P* value of < 0.05 was considered as significant). For gene expression analysis, normal distribution was found using Kolmogorov-Smirnov test, therefore Student's *t*-test (in case of differentiation of two groups with equal variances) or Welch-test (in case of differentiation of two groups with unequal variances) and ANOVA (when more than two groups were compared) were applied. For paired comparisons Benjamini and Hochberg correction was applied. In case of ANOVA, Tukey HSD post-test was used in order to find out which group refers to the differentiation if any. Significance criteria were $P < 0.05$ in any cases. For the logFC calculation, the differences between the averages of groups were considered (abs logFC $\geqslant$ 0.5 criteria).

Methyl capture sequencing - in silico data analysis

Whole methylome data from 6 normal adjacent tissue (NAT), 15 adenoma and 9 CRC tissue samples were determined in a previous study using methyl capture sequencing[12]. Using this dataset, the whole promoter methylation status of genes showing an inverse relation between gene expression and DNA methylation was evaluated. Differentially methylated genes were determined as described earlier[12]. For statistical evaluation normal distribution was determined and the applied tests were chosen according to the above-mentioned criteria. Differences with $P < 0.05$ were considered as significant. Methylation alterations between diagnostic groups were characterized by $\Delta\beta$-values (the differences of the average β-values of sample groups).

Clinical samples

All patients provided informed consent. Colorectal biopsy samples were obtained during routine endoscopic intervention at the 2nd Department of Internal Medicine and 1st Department of Paediatrics, Semmelweis University, Budapest, Hungary. In total 55 colonic tissue samples (from 19 healthy children (under age of 18 years), 8 healthy adults, 20 patients with adenomas and 8 CRC samples) were tested in SFRP1 methylation-specific high resolution melting (MS-HRM) study (Table 1). Biopsy samples from all adults and 5 of children were stored in RNALater Stabilization Solution (Ambion, ThermoFisher Scientific) at -80 ℃ until use. Biopsy samples from the same site were immediately fixed in buffered formalin for histological evaluation. For 14 children, only FFPE blocks were available. Histological diagnoses were established by experienced pathologists. Altogether 27 tissue samples (19 from children and 8 from adults) with normal histology (so called healthy normal colonic tissue samples) were involved in SFRP1 MS-HRM study. Children and adults in the study had been referred to the outpatient clinic with rectal bleeding, constipation or chronic abdominal pain. Ileocolonoscopy was part of their diagnostic procedure (exclude organic disease) and the biopsy specimens showed normal macroscopic appearance and histology[28]. The study was conducted according to the Helsinki declaration and approved by the local ethics committee and government authorities (Regional and Institutional Committee of Science and Research Ethics (TUKEB) Nr.: 69/2008, 202/2009 and 23970/2011 Semmelweis University, Budapest, Hungary).

DNA isolation

Tissue samples were homogenized in 2% sodium dodecyl sulphate, and digested with 4 mg/mL proteinase K for 16 h at 56 ℃. Genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's instructions[18].

Table 1 Clinical data of samples involved in the high resolution melting study

Sample ID	Age	Gender	Localization	Histology	TNM	Grade	Dukes' stage (MAC)	Dysplasia	Adenoma size	Sample type
Ch1	4	M	Colon	Normal						FFPE
Ch2	7	F	Cecum	Normal						FFPE
Ch3	11	M	Colon	Normal						FFPE
Ch4	14	M	Transverse	Normal						FFPE
Ch5	5	F	Sigmoid	Normal						FFPE
Ch6	7	F	Descendent	Normal						FFPE
Ch7	1	M	Descendent	Normal						FFPE
Ch8	1	M	Sigmoid	Normal						FFPE
Ch9	10	M	Cecum	Normal						FFPE
Ch10	3	M	Sigmoid	Normal						FFPE
Ch11	17	F	Cecum	Normal						FFPE
Ch12	17	F	Sigmoid	Normal						FFPE
Ch13	16	F	Sigmoid	Normal						FFPE
Ch14	16	F	Cecum	Normal						FFPE
Ch15	1	M	Left colon	Normal						FF
Ch16	3	F	Sigmoid	Normal						FF
Ch17	6	M	Sigmoid	Normal						FF
Ch18	9	M	Sigmoid	Normal						FF
Ch19	17	M	Colon	Normal						FF
N1	44	F	Sigmoid	Normal						FF
N2	31	F	Sigmoid	Normal						FF
N3	59	F	Sigmoid	Normal						FF
N4	54	M	Colon	Normal						FF
N5	68	F	Sigmoid	Normal						FF
N6	71	F	Sigmoid	Normal						FF
N7	69	F	Sigmoid	Normal						FF
N8	57	F	Sigmoid	Normal						FF
AD1	78	M	Asc, sigmoid, rectum	Tubulovillous adenoma				Low-grade	30 mm, 3 mm, 15 mm	FF
AD2	60	M	Sigmoid	Tubular adenoma				Low-grade	6 mm	FF
AD3	88	M	Asc, transv, sigmoid	Tubular adenoma				Low-grade	4 mm, 3 mm, 7-8 mm	FF
AD4	72	F	Rectum	Tubular adenoma				Low-grade	10 mm	FF
AD5	45	F	Descendent	Tubular adenoma				High-grade	5-6 mm	FF
AD6	68	F	Rectum	Tubular adenoma				Low-grade	5 mm	FF
AD7	63	F	Sigmoid	Tubular adenoma				Low-grade	8 mm	FF
AD8	65	F	Asc, transv, rectum	Tubular adenoma				Low-grade	2-3 mm, 2-3 mm, 2-3 mm	FF
AD9	60	F	Sigmoid	Tubular adenoma				Low-grade	5 mm, 4 mm	FF
AD10	77	F	Rectosigmoid	Tubular adenoma				Low-grade	5 mm	FF
AD11	55	F	Asc colon	Tubular adenoma				Low-grade	10 mm	FF
AD12	76	M	Cecum, sigmoid	Tubular adenoma				Low-grade	5 mm, 8-10 mm	FF
AD13	62	F	Sigmoid	Tubular adenoma				High-grade	30 mm	FF
AD14	83	M	Asc colon	Tubulovillous adenoma				High-grade	50-60 mm	FF
AD15	73	M	Cecum, asc, desc	Tubular adenoma				Low-grade	12 mm, 10 mm, 6-8 mm	FF
AD16	64	M	Transv, sigmoid, rectum	Tubular adenoma				Low-grade	5 mm, 25 mm, 15 mm	FF
AD17	63	M	Asc, transv, rectum	Tubular adenoma				Low-grade	2-3 mm, 5 mm, 5-6 mm, 15 mm	FF
AD18	63	F	Sigmoid	Tubulovillous adenoma				Low-grade	25 mm	FF
AD19	63	M	Rectum	Tubulovillous and tubular adenoma				Low-grade	25 mm, 30 mm	FF

AD20	87	M	Sigmoid, rectum	Tubulovillous adenoma				Low-grade	20 mm, 15 mm	FF
AD21	63	F	Sigmoid	Tubulovillous adenoma				Low-grade	25 mm	FF
CRC1	67	F	Sigmoid	Adenocarcinoma	Unknown	Unknown	Unknown			FF
CRC2	76	F	Hepatic flexure	Adenocarcinoma	T2N0M0	2	B1			FF
CRC3	73	F	Sigmoid	Adenocarcinoma	T3N2M1	2	D			FF
CRC4	65	M	Sigmoid	Adenocarcinoma	T2N0M0	1	B1			FF
CRC5	85	F	Cecum	Adenocarcinoma	T2N0M0	2	B1			FF
CRC6	60	M	Hepatic flexure	Adenocarcinoma		unknown	D			FF
CRC7	68	M	Sigmoid, rectum	Adenocarcinoma	T3N0M0	1	B2			FF
CRC8	87	F	Sigmoid	Adenocarcinoma	T3N0M0	2	B2			FF

Ch: Children; N: Normal; Ad: Adenoma; CRC: Colorectal cancer.

DNA was eluted in 2 × 100 μL RNase- and DNase-free water and stored at -20 ℃. The quantity of the isolated DNA samples was measured by Qubit fluorometer using the Qubit dsDNA HS Assay (Invitrogen, ThermoFisher Scientific).

Bisulfite-specific PCR and MS-HRM experiments for SFRP1 promoter methylation analysis

Bisulfite conversion was performed using the EZ DNA Methylation Direct™ Kit (Zymo Research, Irvine, CA, United States) according to the manufacturer's instructions. For fresh frozen samples, 500 ng isolated DNA was converted, while for FFPE samples the total recovered DNA after deparaffinization and digestion was bisulfite converted. Bisulfite-specific PCR (BS-PCR) reactions were performed in 15 μL volume using LightCycler 480 Probes Master (Roche Applied Science), LightCycler® 480 ResoLight Dye (Roche), *SFRP1* primers at 0.2 μmol/L final concentrations and bisulfite converted DNA (bcDNA) samples (approx, 5 ng bcDNA/well). The sequences of the applied SFRP1 BS-PCR primers were previously described[19]. Real-time PCR amplification was carried out on LightCycler 480 System with the following thermocycling conditions: 95 ℃ for 10 min, then 95 ℃ for 30 s, 62 ℃ with 0.4 ℃ decreasement/cycle for 30 s, 72 ℃ for 30 s for 10 touchdown cycles, followed by the amplification at 95 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s in 50 cycles. For HRM calibration, unmethylated and methylated bisulfite converted control DNA (EpiTect PCR Control DNA Set, Qiagen) were used in different ratios (0%, 10%, 25%, 50%, 75% and 100% methylated controls). HRM analysis was performed according to the following thermal conditions: after denaturation at 95 ℃ for 1 min, and cooling at 40 ℃ for 1 min, the reactions were continuously warmed up to 95 ℃ with a 25 acquisition/℃ rate. Raw HRM data were exported and the HRM peak heights of the negative derivative of fluorescence over temperature curves (-(d/dT) Fluorescence) of the biological samples were retrieved at the melting temperatures of the methylated and unmethylated standards. The methylation percent was calculated by the ratio of values at the methylated and unmethylated melting temperatures. For statistical evaluation normal distribution was determined and the applied tests were chosen according to the above-mentioned criteria.

SFRP1 immunohistochemistry

Parallel with our epigenetic examinations, 4 nm thick FFPE samples from healthy children (n = 6) and healthy adults (n = 7) were examined. SFRP1 immunohistochemistry was performed on colonic tissue samples of healthy adults (n = 7; mean age at histology examination: 48 ± 17 years; 5 f/2 m) and of children (mean age: 12 ± 6 years); 3f/3m). Histology was diagnosed by an expert pathologist on routinely stained HE sections. Following deparaffinization and rehydration, microwave based antigen retrieval was performed in TRIS EDTA buffer (pH 9.0) (900 W/10 min, then 340 W/40 min). Samples were immunostained with SFRP1 polyclonal antibody (ab4193, Abcam, Cambridge, United Kingdom, 1:80 dilution) with diaminobenzidine - hydrogen peroxidase - chromogen-substrate system (cat#30014.K, HISTOLS-DAB, Histopathology Ltd., Hungary) and were digitalized by Pannoramic 250 Flash II scanner (with Zeiss Plan-Apochromat 20 × objective; 3DHISTECH Ltd, Hungary). Digital slides were semi-quantitatively analyzed with Pannoramic Viewer (ver.:1.15.3; 3DHISTECH) based on Q-score method (scored by multiplying the percentage of positive cells (P) by the intensity (I: +3, +2, +1, 0). Formula: Q = P × I; Maximum = 300). Epithelial and stromal compartments were examined separately, then these scores were summarized (Σ) (Σ Q-score maximum: 600) in order to have comparable results with our whole biopsy methylation analyses.

Statistical analysis

The applied statistical methods are outlined above after the descriptions of molecular and *in silico* analyses. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Gene ontology of 353 CpG sites of the "epigenetic clock"

The "epigenetic clock" signature includes 353 CpG sites[2] including different genes, gene promoters and

Table 2 Significant DNA methylation alterations of age-related CpG sites in CRC samples compared to normal tissue

cgID	Gene symbol	*P* value	Δβ (CRC - N)
cg06462291	NT5DC3	3.81×10^{-6}	-0.27
cg06144905	PIPOX	1.81×10^{-7}	-0.22
cg10345936	SLC36A2	2.49×10^{-5}	-0.18
cg13828047	MPI	1.78×10^{-5}	-0.17
cg11314684	AKT3	4.55×10^{-5}	-0.16
cg00431549	MGP	0.020	-0.12
cg14409958	ENPP2	0.021	-0.11
cg00091693	KRT20	0.010	-0.11
cg17274064	ERG	1.48×10^{-4}	-0.11
cg22809047	RPL31	1.91×10^{-3}	-0.10
cg00075967	STRA6	0.010	-0.10
cg06952310	NCAN	7.13×10^{-3}	-0.10
cg02388150	SFRP1	3.33×10^{-3}	0.10
cg03588357	GPR68	7.72×10^{-3}	0.10
cg26297688	TMEM263	6.21×10^{-3}	0.10
cg04528819	KLF14	0.036	0.10
cg26372517	TFAP2E	9.23×10^{-3}	0.11
cg08030082	POMC	1.93×10^{-3}	0.11
cg05675373	KCNC4	7.80×10^{-3}	0.11
cg10281002	TBX5	0.012	0.11
cg06117855	CLEC3B	1.87×10^{-3}	0.11
cg09509673	CCR10	1.91×10^{-4}	0.11
cg14597908	GNAS	1.48×10^{-4}	0.11
cg27494383	LTK	9.32×10^{-3}	0.12
cg21870884	GPR25	5.77×10^{-3}	0.13
cg25657834	NTSR2	4.43×10^{-3}	0.13
cg22449114	TCF15	3.48×10^{-5}	0.13
cg04126866	C10orf99	4.52×10^{-4}	0.13
cg25552492	LGI3	9.44×10^{-3}	0.14
cg08965235	LTBP3	3.12×10^{-3}	0.14
cg06836772	PRKAA2	0.013	0.14
cg02364642	ARHGEF25	1.20×10^{-3}	0.16
cg18573383	KCNC2	1.38×10^{-3}	0.16
cg12616277	ESYT3	0.015	0.16
cg17729667	NINL	3.46×10^{-3}	0.16
cg04999691	ZBED6CL	3.32×10^{-11}	0.16
cg12373771	CECR6	3.70×10^{-3}	0.17
cg02489552	CCDC105	8.91×10^{-8}	0.17
cg25148589	GRIA2	7.77×10^{-6}	0.19
cg21096399	MCAM	5.47×10^{-6}	0.19
cg20914508	GAP43	4.24×10^{-4}	0.20
cg12768605	LYPD5	1.85×10^{-4}	0.21
cg13216057	DKK3	2.10×10^{-4}	0.21
cg12351433	LHCGR	4.97×10^{-7}	0.22
cg08434234	DGKI	5.16×10^{-5}	0.23
cg10920957	JPH3	3.00×10^{-4}	0.26
cg27092035	ARL10	3.73×10^{-5}	0.27
cg06557358	TMEM132E	3.17×10^{-6}	0.27
cg26620959	SYNE1	1.32×10^{-6}	0.30
cg07663789	NPR3	8.73×10^{-6}	0.31
cg09191327	PRDM12	1.79×10^{-6}	0.31
cg25070637	SDC2	1.64×10^{-6}	0.34
cg10486998	GALR1	1.81×10^{-8}	0.35
cg24834740	PPP1R16B	3.56×10^{-12}	0.37
cg27319898	ZNF804B	5.41×10^{-8}	0.38
cg08090772	ADHFE1	7.18×10^{-14}	0.39
cg02217159	KHDRBS2	6.67×10^{-13}	0.43

N: Normal; CRC: Colorectal cancer.

other genomic regions such as enhancers, insulators, Polycomb-repressed regions. From the above 353 CpG sites, DNA methylation levels of 193 were positively and of 160 were negatively correlated with age[2].

First we updated the annotation of the CpG sites and assigned official gene symbols according to the newest version of NCBI Gene Database. Approximately 80% of the genes belonging to 353 CpG sites could be classified into functional groups including highly represented transcriptional regulation, translation (15.93%), metabolism (12.36%), development and ontogenesis (8.24%) and transport (8.24%). Approximately 20% of the genes had unknown function. According to the Encode ChromHMM results of nine human cell lines, the majority of the 353 CpG sites (76.2 %) were located in the promoter regions of genes. Also, 57.2% of them were categorized as active promoters (declared if it was found "active promoter" in at least one of the nine analyzed cell lines), while 19% were found to be "weak promoter" ("weak promoter" in at least one of the nine analyzed cell lines). One fourth (23.8%) of the CpG sites was located in non-promoter regions such as enhancers, insulators, transcribed and repressed regions (Supplementary Table 1).

In silico DNA methylation analysis

Analysis of the Illumnina Beadchip450K methylation array data set of Luo *et al*[24] showed 137 (38.8%) of the epigenetic clock CpG sites were found to be significantly differentially methylated between CRC and normal tissue samples ($P < 0.05$). Approximately two third of these CpG sites had similar methylation changes in CRC samples as during aging, while one third of these CpG sites showed opposite alterations in CRC tissue as during aging (Supplementary Table 1). Among these, 57 CpG sites showed at least a 10% methylation difference: from the 57 CpG sites 45 were hypermethylated (including *ADHFE1*/cg08090772, *MCAM*/cg21096399, *DKK3*/cg13216057, *SFRP1*/cg02388150, *SYNE1*/cg26620959), while 12 CpG sites showed hypomethylation (such as *MGP*/cg00431549, *PIPOX*/cg06144905, *STRA6*/cg00075967, *ERG*/cg17274064) in CRCs ($P < 0.05$, $\Delta\beta \geqslant 10\%$) (Table 2, Supplementary Table 1). In the adenoma *vs* normal comparison, DNA methylation of 165 CpG sites (46.7%) were significantly altered from which 70 CpG sites showed a $\geqslant 10\%$ methylation difference: 36 CpG sites were hypermethylated (*e.g.*, *SDC2*/cg25070637, *SFRP1*/cg02388150, *SYNE1*/cg26620959) and 34 showed decreased methylation levels (including *CEMIP*/cg20828084, *SPATA18*/cg03103192, *STRA6*/cg00075967) in adenoma samples ($P < 0.05$, $\Delta\beta \geqslant 10\%$) (Table 3). In CRC samples 33 CpG sites were found to be hypermethylated (such as *KRT20*/cg00091693, *STRA6*/cg00075967, *UROS*/cg19346193) and only one (*LTBP3*/cg08965235) was hypomethylated compared to adenomas ($P < 0.05$, $\Delta\beta \geqslant 10\%$) (Table 4). A heatmap for the differentially methylated epigenetic clock CpG sites ($P < 0.05$, $\Delta\beta \geqslant 10\%$), with hierarchical cluster analysis results of normal, adenoma and CRC samples is shown in Figure 1.

Table 3 Significant DNA methylation changes of age-related CpG sites in adenoma samples compared to normal tissue

cgID	Gene symbol	*P* value	Δβ (AD - N)
cg10345936	SLC36A2	3.49×10^{-10}	-0.34
cg00091693	KRT20	2.49×10^{-12}	-0.30
cg00075967	STRA6	2.58×10^{-9}	-0.26
cg11314684	AKT3	1.03×10^{-9}	-0.26
cg06462291	NT5DC3	7.45×10^{-5}	-0.25
cg17099569	GLI2	1.25×10^{-10}	-0.23
cg00168942	GJD4	2.48×10^{-7}	-0.20
cg20828084	CEMIP	1.33×10^{-9}	-0.19
cg17274064	ERG	1.82×10^{-10}	-0.19
cg07408456	PGLYRP2	2.41×10^{-12}	-0.19
cg03019000	TEX264	2.25×10^{-7}	-0.19
cg17589341	SLC14A1	1.42×10^{-9}	-0.19
cg02580606	KRT33B	9.08×10^{-5}	-0.17
cg00436603	CYP2E1	1.37×10^{-8}	-0.16
cg19346193	UROS	6.59×10^{-7}	-0.15
cg03103192	SPATA18	1.39×10^{-7}	-0.15
cg25564800	KPNA1	2.18×10^{-8}	-0.14
cg06144905	PIPOX	3.63×10^{-7}	-0.14
cg06952310	NCAN	2.72×10^{-4}	-0.14
cg13038560	C2orf47	2.13×10^{-5}	-0.13
cg22190114	NLRP8	1.67×10^{-4}	-0.13
cg13302154	MGP	7.27×10^{-6}	-0.13
cg01262913	DSCR9	2.67×10^{-6}	-0.13
cg14258236	OR5V1	3.71×10^{-4}	-0.12
cg13828047	MPI	1.01×10^{-3}	-0.12
cg01459453	SELP	1.65×10^{-4}	-0.12
cg14894144	LAMA3	2.29×10^{-4}	-0.12
cg07337598	ANXA9	2.02×10^{-5}	-0.12
cg03270204	DDR1	7.47×10^{-5}	-0.12
cg12946225	HMG20B	1.37×10^{-6}	-0.11
cg09646392	TNFSF13B	2.66×10^{-5}	-0.10
cg19305227	SLC28A2	0.033	-0.10
cg03578041	LARP6	2.22×10^{-5}	-0.10
cg07455279	NDUFA3	0.013	-0.10
cg13899108	PDE4C	5.00×10^{-6}	0.10
cg04999691	ZBED6CL	4.92×10^{-5}	0.10
cg14597908	GNAS	9.38×10^{-5}	0.10
cg21870884	GPR25	9.49×10^{-3}	0.11
cg12616277	ESYT3	0.022	0.12
cg12373771	CECR6	3.55×10^{-3}	0.12
cg17729667	NINL	4.43×10^{-3}	0.13
cg02364642	ARHGEF25	2.33×10^{-3}	0.14
cg22449114	TCF15	2.39×10^{-5}	0.14
cg03565323	ZNF287	4.52×10^{-3}	0.14
cg02388150	SFRP1	1.20×10^{-7}	0.15
cg12768605	LO×L2	8.01×10^{-3}	0.15
cg25657834	NTSR2	1.46×10^{-4}	0.16
cg20914508	GAP43	9.41×10^{-4}	0.16
cg10281002	TBX5	1.26×10^{-5}	0.17
cg02489552	CCDC105	7.16×10^{-8}	0.18
cg05675373	KCNC4	1.82×10^{-6}	0.18
cg25148589	GRIA2	6.11×10^{-7}	0.18
cg13216057	DKK3	9.64×10^{-4}	0.18
cg27092035	ARL10	8.81×10^{-4}	0.19
cg12351433	LHCGR	2.20×10^{-6}	0.20
cg18573383	KCNC2	5.43×10^{-7}	0.21
cg08434234	DGKI	6.60×10^{-5}	0.22
cg21096399	MCAM	5.20×10^{-8}	0.22
cg10920957	JPH3	1.03×10^{-4}	0.25
cg08965235	LTBP3	6.03×10^{-10}	0.25
cg27319898	ZNF804B	5.48×10^{-5}	0.26
cg26620959	SYNE1	3.89×10^{-6}	0.29
cg25070637	SDC2	1.82×10^{-5}	0.30
cg07663789	NPR3	6.41×10^{-7}	0.31
cg09191327	PRDM12	4.08×10^{-7}	0.31
cg06557358	TMEM132E	2.04×10^{-8}	0.33
cg10486998	GALR1	2.61×10^{-9}	0.36
cg24834740	PPP1R16B	2.23×10^{-9}	0.36
cg08090772	ADHFE1	5.19×10^{-11}	0.39
cg02217159	KHDRBS2	1.87×10^{-11}	0.41

N: Normal; Ad: Adenoma.

Table 4 Significant DNA methylation alterations of age-related CpG sites in colorectal cancer samples compared to adenoma tissue

cgID	Gene symbol	*P* value	Δβ (CRC - AD)
cg08965235	LTBP3	9.09×10^{-4}	-0.11
cg00945507	SEC61G	1.64×10^{-5}	0.10
cg15974053	HSD17B14	2.27×10^{-3}	0.10
cg24262469	TIPARP	5.33×10^{-8}	0.10
cg07158339	FXN	1.84×10^{-7}	0.10
cg11314684	AKT3	4.52×10^{-4}	0.10
cg03578041	LARP6	2.57×10^{-9}	0.10
cg17853587	NDST3	5.94×10^{-4}	0.10
cg07408456	PGLYRP2	1.51×10^{-7}	0.11
cg02580606	KRT33B	1.22×10^{-4}	0.11
cg03019000	TEX264	5.39×10^{-5}	0.11
cg27319898	ZNF804B	0.023	0.12
cg00436603	CYP2E1	1.97×10^{-6}	0.12
cg15804973	MAP3K5	6.27×10^{-12}	0.12
cg06117855	CLEC3B	1.73×10^{-7}	0.13
cg24126851	DCHS1	3.58×10^{-8}	0.13
cg16034652	UNC79	4.23×10^{-11}	0.13
cg01262913	DSCR9	1.64×10^{-8}	0.13
cg26372517	TFAP2E	9.80×10^{-6}	0.13
cg03270204	DDR1	4.24×10^{-7}	0.13
cg17589341	SLC14A1	7.35×10^{-10}	0.13
cg22679120	SNX8	8.16×10^{-9}	0.14
cg20828084	CEMIP	2.00×10^{-8}	0.14
cg19305227	SLC28A2	1.11×10^{-6}	0.14
cg00168942	GJD4	1.18×10^{-7}	0.14
cg13038560	C2orf47	8.96×10^{-8}	0.15
cg26614073	SCAP	1.33×10^{-9}	0.15
cg19346193	UROS	2.09×10^{-8}	0.16
cg00075967	STRA6	4.87×10^{-7}	0.16
cg03103192	SPATA18	6.07×10^{-12}	0.16
cg10345936	SLC36A2	4.09×10^{-6}	0.16
cg17099569	GLI2	9.04×10^{-7}	0.16
cg04126866	C10orf99	4.23×10^{-10}	0.18
cg00091693	KRT20	1.17×10^{-8}	0.19

N: Normal; Ad: Adenoma; CRC: Colorectal cancer.

In silico gene expression analysis

Genes belonging to 353 age-related CpG sites were mapped to 768 Affymerix transcript IDs. In the CRC *vs* N comparison, 215 "epigenetic clock" genes were found to be significantly differentially expressed ($P < 0.05$), of which 117 were altered with absolute logFC > 0.5 (70 upregulated such as *ERG*, *MGP*, *MCAM*, *CEMIP* and 47 downregulated like *SFRP1*, *KRT20*, *CLEC3B*, *SYNE1*) (Supplementary Table 2A). Expression of 196 "epigenetic clock" genes changed significantly ($P < 0.05$) in adenoma samples compared to healthy normal controls, 102 with absolute logFC higher than 0.5 (47 overexpressed such as *CEMIP*, *PLK1*, *CCNF* and 55 underexpressed like *SFRP1*, *SDC2*, *SYNE1*) (Supplementary Table 2B). Forty-three genes including *MCAM*, *MGP* and *AKT3* showed increased expression in

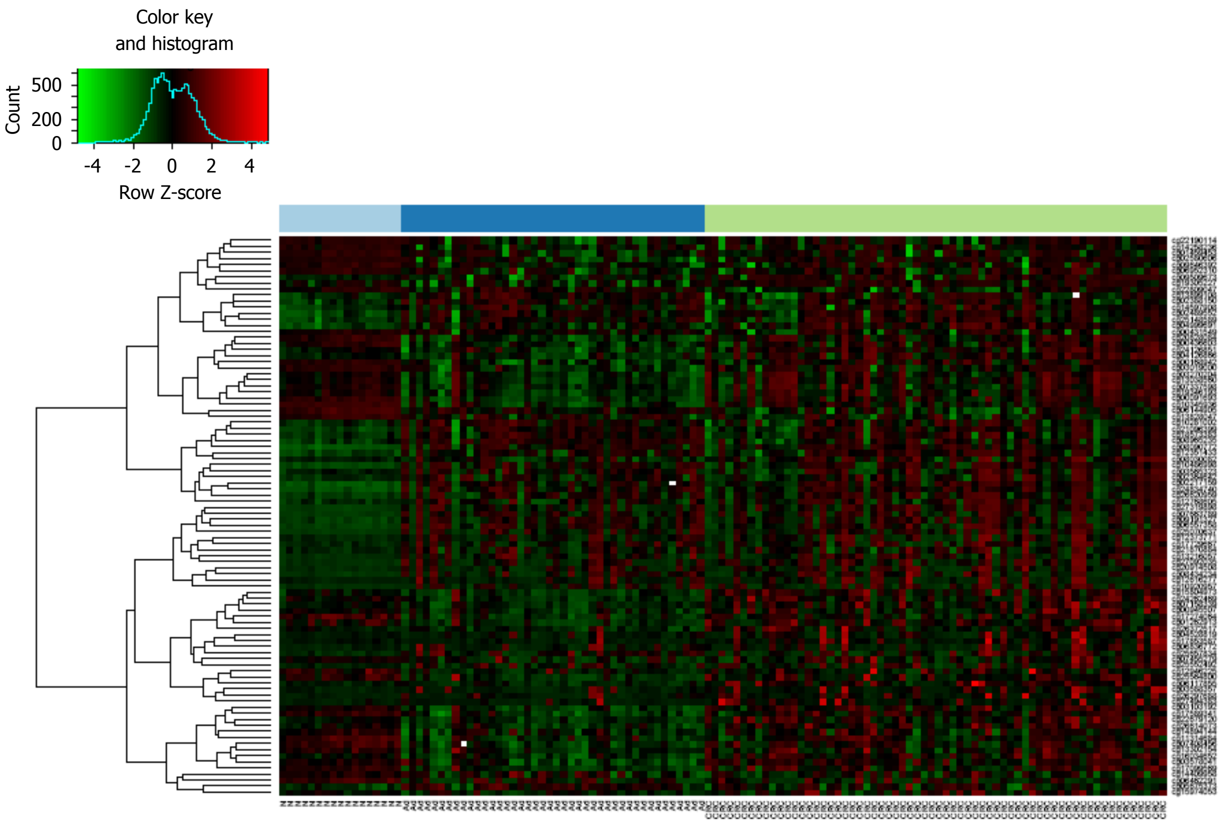

Figure 1 DNA methylation heatmap of normal, adenoma and colorectal carcinoma samples according to the methylation status of age-related CpG sites. From the 353 epigenetic clock CpG sites (cg IDs)[2] significantly differentially methylated in CRC *vs* normal, adenoma *vs* normal and CRC *vs* adenoma comparisons were selected and colonic tissue samples (GSE48684[24]) were classified according to their methylation levels. The analyzed samples are illustrated on X axis, significantly altered CpG sites (cg IDs) are represented on Y axis. DNA methylation intensities (β values) are visualized, red shows hypermethylation, while hypomethylation was marked with green color. CRC: Colorectal carcinoma (light green); Ad: Adenoma (dark blue); N: Normal (light blue).

CRC compared to adenoma samples, while 17 genes including *CEMIP*, *SPATA18* were downregulated (P < 0.05, absolute logFC > 0.5) (Supplementary Table 2C).

For genes with an inverse relation between gene expression and promoter methylation, the genes with both significant mRNA expression changes with absolute logFC > 0.5 and significant DNA methylation alterations with at least 10% difference were taken into consideration. Based on these criteria, eleven genes, including *ERG*, *MGP*, *PIPOX*, *CLEC3B*, *LTK*, *SFRP1* and *SYNE1* were found to be inversely expressed with the promoter methylation status in CRC compared to normal tissue. Compared to the promoter methylation alterations, the expression of 8 genes, including *CEMIP*, *SPATA18*, *SDC2*, *SFRP1* and *SYNE1* changed oppositely in AD *vs* N comparison, while in CRC *vs* AD tissues 3 genes, namely *CEMIP*, *SPATA18* and *SLC28A2* showed this expression pattern. The genes showing an inverse relation between gene expression and DNA methylation in CRC *vs* normal, AD *vs* normal and CRC *vs* AD comparisons are represented in Table 5.

In the comparison of healthy young colonic samples and normal adult tissues, 150 genes showed significantly altered expression from which 94 genes with absolute logFC > 0.5 including overexpressed *LTBP3*, *REEP1*, *MGP*, *PLK1*, *SFRP1*, *SYNE1* and downregulated *PRKG2*, *PDCD6IP* and *TMEM56* (P < 0.05) (Supplementary Table 2D). The pattern of expression of several genes including *SYNE1*, *CLEC3B*, *LTBP3* (Figure 2) and *SFRP1* (Figure 3A-C) was similar for increased age as that observed for cancer progression. However, there were some genes such as *MGP* (Figure 2) which showed similar expression pattern in young people and in cancer patients compared to healthy adult tissue.

Whole promoter methylation status of genes showing inverse relationship between gene expression and DNA methylation

The DNA methylation status of whole promoter regions of genes showing an inverse relation between gene expression and DNA methylation (Table 5) was determined using methyl capture sequencing data of 6 NAT, 15 adenoma and 9 CRC tissue samples[12]. In the CRC *vs* N/NAT comparison, similar DNA methylation alterations (such as hypomethylated *AKT3*, *MGP* promoters and hypermethylated *PPP1R16B*, *SFRP1*, *SYNE1* promoters) were observed in the promoter regions of 7 of the 11 inversely expressed genes (Table 6). Between adenoma and normal samples, promoter regions of 7 of the 8 inversely expressed genes showed DNA methylation differences (*e.g.*,

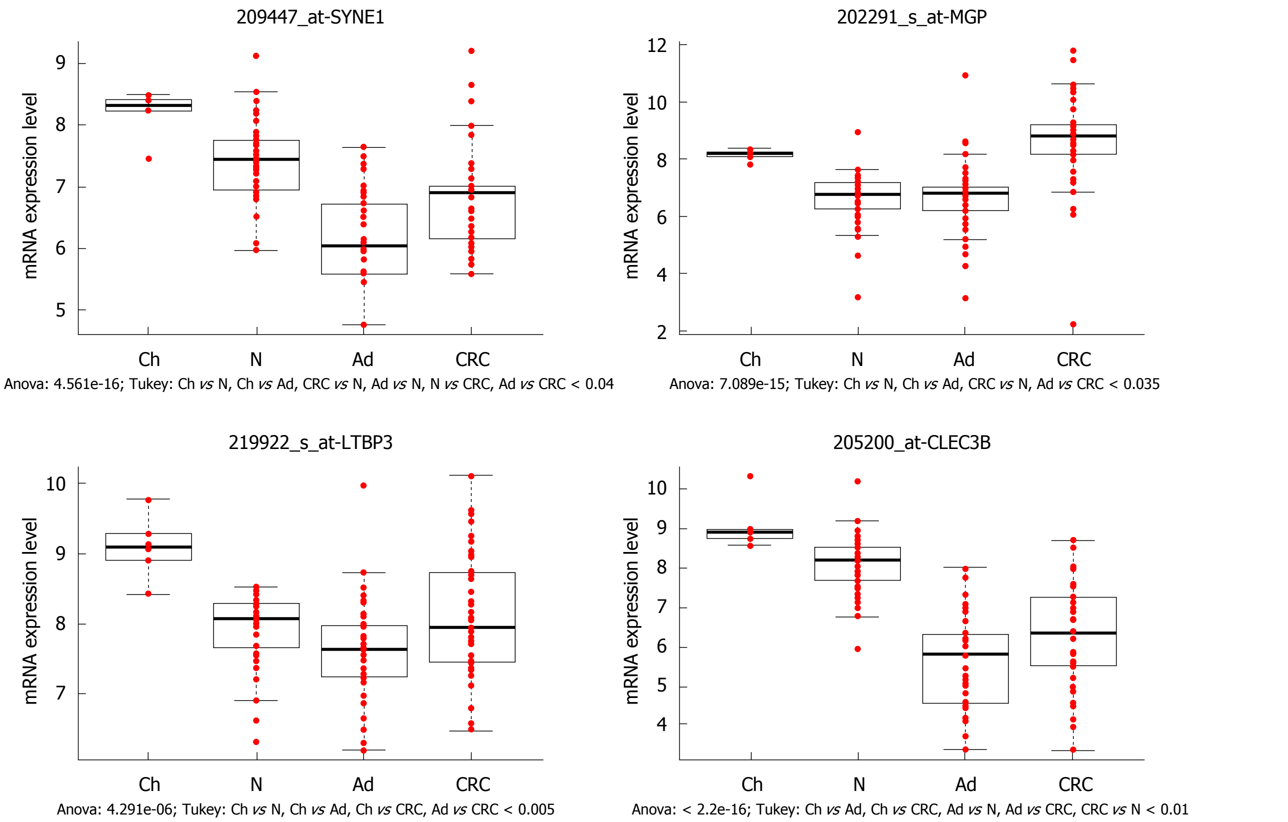

Figure 2 Genes showing both age- and carcinogenesis-related expression alterations. SYNE1 (spectrin repeat containing nuclear envelope protein 1), LTBP3 (latent transforming growth factor beta binding protein 3) and CLEC3B (C-type lectin domain family 3 member B) genes were downregulated during the colorectal carcinogenesis, similar decreasing expression was found during aging (significantly higher mRNA levels were detected in young colonic samples than in healthy adult biopsy specimens). MGP (matrix Gla protein) was overexpressed in children and in CRC samples compared to adenoma and healthy adults ($P < 0.035$), hence its opposite expression was found during aging and colorectal carcinogenesis. X axis shows the analyzed sample groups, the normalized mRNA expression can be seen on Y axis. Red dots indicate the normalized mRNA expression values, boxplots represent the medians and standard deviations. Ch: Children; N: Normal; Ad: Adenoma; CRC: Colorectal cancer.

hypomethylated *CEMIP/KIAA1199*, *SPATA18* promoters and hypermethylated *DKK3*, *SDC2*, *SFRP1*, *SYNE1* promoters) (Table 6) as detected in case of age-related CpG sites. In CRC samples compared to adenoma tissue, significant hypermethylation of *CEMIP/KIAA199* promoter could be demonstrated in "epigenetic clock" CpG sites and whole promoter methylation analyses (Table 6).

SFRP1 promoter methylation analysis in healthy children, healthy adult, adenoma and CRC tissues

Based on the gene expression analysis results, *SFRP1* was found to be overexpressed in normal adult samples compared to adenoma and CRC biopsy specimens, and in healthy young patients even higher *SFRP1* mRNA levels could be detected than in normal adult samples (Figure 3A-C). As *SFRP1* is proven to be a methylation-regulated gene with literature data regarding its age-related DNA methylation alterations, hence it was chosen for detailed methylation analysis of normal, premalignant and cancerous colonic specimens including tissue samples from healthy children.

SFRP1 promoter sequences were highly methylated in CRC samples (average methylation% = 55.0% ± 8.4%) and in adenoma tissue (49.9% ± 18.1 %), while low methylation levels could be measured in colonic tissues of both healthy adults (5.2% ± 2.7%) and children (2.2% ± 0.7%). Significant considerable hypermethylation was found in *SFRP1* promoter both between CRC and adult normal and between CRC and healthy children colonic tissue samples ($P < 0.0001$) (Figure 3D). In the healthy adult *vs* healthy children comparison, significant, but moderate DNA methylation alterations were detected: in adults higher DNA methylation levels were found in the analyzed region of *SFRP1* promoter ($P = 0.017$) (Figure 3E).

SFRP1 protein expression in colonic tissue samples of healthy normal children and adults

In healthy children samples the epithelial layer showed strong (representative scoring values: +3 and +2), diffuse SFRP1 expression both in cytoplasmic and nuclear region (Q-score: 226.67 ± 17.51), whereas the stromal cells showed heterogeneous protein expression (scoring values: form +3 to 0; Q-score: 176.66 ± 18.61; ΣQ-score: 403.33 ± 22.51; Figure 4A). Among stromal cells subepithelial fibroblast and several immune cells showed strong cytoplasmic

Table 5 Genes showing inverse DNA methylation and gene expression data

Gene symbol	Gene name	DNA methylation			Gene expression		
		cg ID	*P* value	Δβ	Affymetrix ID	*P* value	LogFC
CRC *vs* N							
AKT3	AKT serine/threonine kinase 3	cg11314684	4.55×10^{-5}	-0.16	224229_s_at	0.015	0.07
					222880_at	2.26×10^{-3}	0.61
					212609_s_at	4.72×10^{-4}	0.62
					212607_at	9.76×10^{-4}	0.64
ERG	v-ets avian erythroblastosis virus E26 oncogene homolog	cg17274064	1.48×10^{-4}	-0.11	211626_x_at	5.84×10^{-4}	0.20
					241926_s_at	2.05×10^{-5}	0.64
					213541_s_at	4.69×10^{-9}	1.08
MGP	Matrix Gla protein	cg00431549	0.020	-0.12	202291_s_at	2.34×10^{-11}	1.97
PIPOX	Pipecolic acid and sarcosine oxidase	cg06144905	1.81×10^{-7}	-0.22	221605_s_at	0.019	0.50
CCR10	C-C motif chemokine receptor 10	cg09509673	1.91×10^{-4}	0.11	220565_at	0.010	-0.51
CLEC3B	C-type lectin domain family 3 member B	cg06117855	1.88×10^{-3}	0.11	205200_at	1.45×10^{-13}	-1.79
LTK	Leukocyte receptor tyrosine kinase	cg27494383	9.32×10^{-3}	0.12	217184_s_at	2.14×10^{-11}	-1.32
					207106_s_at	2.00×10^{-5}	-0.75
PPP1R16B	Protein phosphatase 1 regulatory subunit 16B	cg24834740	3.56×10^{-12}	0.37	41577_at	1.76×10^{-4}	-0.79
					212750_at	7.68×10^{-3}	-0.48
PRKAA2	Protein kinase AMP-activated catalytic subunit alpha 2	cg06836772	0.013	0.14	227892_at	4.15×10^{-3}	-0.87
					238489_at	4.67×10^{-3}	-0.22
SFRP1	Secreted frizzled related protein 1	cg02388150	3.33×10^{-3}	0.10	202036_s_at	8.66×10^{-8}	-1.47
					202037_s_at	3.98×10^{-5}	-1.11
					202035_s_at	0.039	-0.28
SYNE1	Spectrin repeat containing nuclear envelope protein 1	cg26620959	1.32×10^{-6}	0.30	209447_at	4.27×10^{-5}	-0.63
AD *vs* N							
CEMIP	Cell migration inducing hyaluronan binding protein	cg20828084	1.33×10^{-9}	-0.17	1554685_a_at	1.71×10^{-11}	1.12
					212942_s_at	2.41×10^{-43}	6.88
NT5DC3	5'-nucleotidase domain containing 3	cg06462291	7.45×10^{-5}	-0.25	218786_at	6.83×10^{-9}	0.76
SPATA18	Spermatogenesis associated 18	cg03103192	1.39×10^{-7}	-0.15	230723_at	4.41×10^{-5}	0.45
					229331_at	1.20×10^{-10}	1.50
DKK3	Dickkopf WNT signaling pathway inhibitor 3	cg13216057	9.64×10^{-4}	0.18	202196_s_at	9.82×10^{-5}	-0.65
					214247_s_at	1.52×10^{-4}	-0.50
PPP1R16B	Protein phosphatase 1 regulatory subunit 16B	cg24834740	2.23×10^{-9}	0.36	41577_at	1.20×10^{-10}	-1.44
					212750_at	3.28×10^{-7}	-0.81
SDC2	Syndecan 2	cg25070637	1.82×10^{-5}	0.30	212158_at	1.74×10^{-10}	-1.17
					212157_at	2.14×10^{-8}	-0.66
					212154_at	9.19×10^{-5}	-0.64
SFRP1	Secreted frizzled related protein 1	cg02388150	1.20×10^{-7}	0.15	202036_s_at	3.62×10^{-13}	-1.79
					202037_s_at	4.43×10^{-11}	-1.48
					202035_s_at	0.016	-0.32
SYNE1	Spectrin repeat containing nuclear envelope protein 1	cg26620959	3.89×10^{-6}	0.29	209447_at	5.76×10^{-15}	-1.19
CRC *vs* AD							
CEMIP	Cell migration inducing hyaluronan binding protein	cg20828084	2.00×10^{-8}	0.14	212942_s_at	9.88×10^{-3}	-1.17
SLC28A2	Solute carrier family 28 member 2	cg19305227	1.11×10^{-6}	0.14	207249_s_at	0.027	-1.24
SPATA18	Spermatogenesis associated 18	cg03103192	6.07×10^{-12}	0.16	229331_at	3.52×10^{-9}	-1.73
					230723_at	7.35×10^{-4}	-0.41

N: Normal; Ad: Adenoma; CRC: Colorectal cancer.

and/or nuclear SFRP1 expression. Not significantly, but remarkably lower (representative scoring value: +2) SFRP1 protein expression was detectable both in epithelial (Q-score: 202.14 ± 24.12) and stromal component (140.71 ± 41.47; Σ Q-score: 351.42 ± 68.66; Figure 4B) of adult persons (*P* values: 0.063, 0.073 and 0.105 respectively).

DISCUSSION

Cancer is considered a primary age-related disease[3], and therefore age-related molecular changes including epigenetic alterations such as epigenetic drift and epigenetic clock[1,2] necessarily show relationship with carcinogenesis-associated differences. Besides global hypomethylation, local, genomic site specific hypermethylation principally in the promoter regions of tumor suppressor genes can occur during both processes[6-8].

In this study, the potential correspondence between age-related and CRC-associated DNA methylation changes was studied using the 353 epigenetic clock CpG sites published by Horvath[2] as a model for age-related DNA methylation changes.

Table 6 Whole promoter methylation status of genes with inverse age-related CpG site DNA methylation and gene expression data

Gene symbol	DNA methylation on age-related CpG site			Whole promoter DNA methylation status		
	cg ID	*P* value	Δβ	Start-Stop position	*P* value	Δβ
CRC *vs* N						
AKT3	cg11314684	4.55×10^{-5}	-0.16	chr1:244005801-244005900	6.56×10^{-3}	-0.33
ERG	cg17274064	1.48×10^{-4}	-0.11	chr21:39871501-39871600	0.044	-0.21
MGP	cg00431549	0.020	-0.12	chr12:15038501-15038600	0.030	-0.27
CCR10	cg09509673	1.91×10^{-4}	0.11	chr17:40835101-40835200	0.045	0.18
				chr17:40835201-40835300	0.022	0.26
PPP1R16B	cg24834740	3.56×10^{-12}	0.37	chr20:37433201-37433300	3.76×10^{-3}	0.35
				chr20:37434701-37434800	0.048	0.40
				chr20:37435301-37435400	0.042	0.37
SFRP1	cg02388150	3.33×10^{-3}	0.10	chr8:41166001-41166100	0.017	0.44
				chr8:41166101-41166200	1.31×10^{-4}	0.56
				chr8:41166201-41166300	3.40×10^{-3}	0.49
				chr8:41166301-41166400	0.014	0.27
				chr8:41166401-41166500	1.28×10^{-3}	0.53
				chr8:41166501-41166600	0.027	0.44
				chr8:41166601-41166700	0.034	0.39
				chr8:41166901-41167000	0.042	0.41
SYNE1	cg26620959	1.32×10^{-6}	0.30	chr6:152622201-152622300	0.027	0.35
				chr6:152957601-152957700	0.032	0.39
				chr6:152957701-152957800	0.043	0.40
				chr6:152958101-152958200	2.86×10^{-4}	0.65
AD *vs* N						
CEMIP	cg20828084	1.33×10^{-9}	-0.17	chr15: 81070701-81070800	0.028	-0.25
SPATA18	cg03103192	1.39×10^{-7}	-0.15	chr4:52915901-52916000	0.042	-0.23
DKK3	cg13216057	9.64×10^{-4}	0.18	chr11:12029701-12029800	3.11×10^{-3}	0.26
				chr11:12029801-12029900	1.67×10^{-3}	0.33
				chr11:12029901-12030000	8.08×10^{-5}	0.56
				chr11:12030001-12030100	0.047	0.15
				chr11:12030401-12030500	7.38×10^{-3}	0.28
				chr11: 12030501-12030600	0.012	0.28
PPP1R16B	cg24834740	2.23×10^{-9}	0.36	chr20:37434201-37434300	0.045	0.26
				chr20:37434601-37434700	0.022	0.39
				chr20:37434701-37434800	0.013	0.39
				chr20:37434801-37434900	6.87×10^{-3}	0.30
				chr20:37434901-37435000	1.70×10^{-3}	0.44
				chr20:37435001-37435100	1.93×10^{-3}	0.45
				chr20:37435101-37435200	6.49×10^{-3}	0.35
				chr20:37435201-37435300	8.93×10^{-4}	0.51
				chr20:37435301-37435400	0.014	0.42
SDC2	cg25070637	1.82×10^{-5}	0.30	chr8:97505901-97506000	2.30×10^{-3}	0.59
				chr8:97506101-97506200	1.96×10^{-4}	0.65
				chr8:97506201-97506300	8.43×10^{-4}	0.63
				chr8:97506301-97506400	0.012	0.49
				chr8:97506401-97506500	0.019	0.47
				chr8:97506601-97506700	3.99×10^{-3}	0.51
				chr8:97506701-97506800	0.021	0.45
				chr8:97506801-97506900	0.014	0.52
				chr8:97507101-97507200	0.015	0.44
				chr8:97507201-97507300	0.010	0.52
				chr8:97507301-97507400	3.47×10^{-3}	0.59
SFRP1	cg02388150	1.20×10^{-7}	0.15	chr8:41165901-41166000	2.30×10^{-3}	0.59
				chr8:41166001-41166100	1.57×10^{-3}	0.59
				chr8:41166101-41166200	3.54×10^{-4}	0.56
				chr8:41166201-41166300	1.49×10^{-4}	0.60
				chr8:41166301-41166400	6.54×10^{-3}	0.27
				chr8:41166401-41166500	1.32×10^{-5}	0.55
				chr8: 41166501-41166600	2.30×10^{-3}	0.60
				chr8:41166601-41166700	5.45×10^{-3}	0.57
				chr8:41166701-41166800	8.15×10^{-3}	0.51
				chr8:41166801-41166900	0.032	0.39
				chr8:41166901-41167000	0.019	0.45
				chr8:41167001-41167100	0.046	0.34
				chr8:41167101-41167200	0.025	0.41
				chr8:41167201-41167300	0.024	0.40

SYNE1	cg26620959	3.89×10^{-6}	0.29	chr6:152702701-152702800	0.012	0.20
				chr6:152702801-152702900	0.035	0.21
				chr6:152957501-152957600	6.05×10^{-4}	0.37
				chr6:152957601-152957700	2.34×10^{-4}	0.60
				chr6:152957701-152957800	0.017	0.51
				chr6:152957801-152957900	1.09×10^{-3}	0.42
				chr6:152957901-152958000	1.03×10^{-4}	0.57
				chr6:152958001-152958100	7.13×10^{-4}	0.43
				chr6:152958101-152958200	1.72×10^{-5}	0.72
CRC *vs* AD						
CEMIP	cg20828084	2.00×10^{-8}	0.14	chr15:81070701-81070800	7.59×10^{-4}	0.31
				chr15:81070801-81070900	0.011	0.36

Only significant promoter DNA methylation alterations are represented in the table. $P < 0.05$.

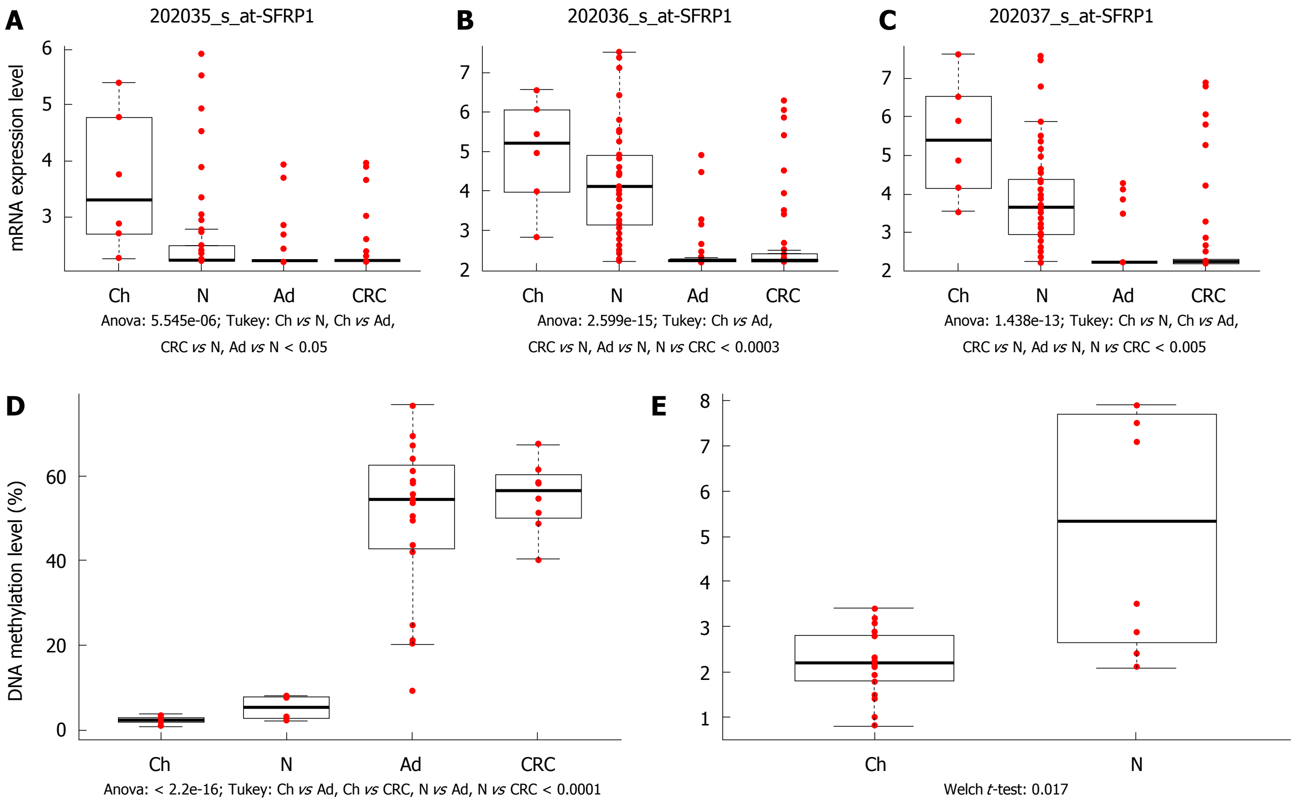

Figure 3 *SFRP1* mRNA expression and promoter DNA methylation alterations during aging and in different stages of colorectal carcinogenesis. *SFRP1* mRNA expression was significantly downregulated in adenoma and CRC samples compared to normal controls in case of all three Affymetrix probeset IDs representing *SFRP1* [202035_s_at: $P < 0.05$ (A); 202036_s_at: $P < 0.0003$ (B); 202037_s_at: $P < 0.005$ (C)]. In colonic biopsy samples of healthy young patients, higher *SFRP1* mRNA levels could be measured than in normal adults samples, this overexpression was proven to be significant in two of three transcript IDs [202035_s_at: $P < 0.05$ (A); 202037_s_at: $P < 0.005$ (C)]. *SFRP1* promoter region was significantly hypermethylated in CRC and adenoma tissue samples compared to normal adult and young colonic tissue ($P < 0.0001$) (D). In pairwise comparison, DNA methylation of *SFRP1* promoter was slightly, but significantly increased in healthy adults compared to normal young samples ($P < 0.02$) (E). The analyzed sample groups are illustrated on X axis, the normalized mRNA expression (A, B, C) and percentage of *SFRP1* promoter methylation (D, E) are represented on Y axis. Red dots indicate the normalized mRNA expression values (A, B, C) and the normalized DNA methylation percentage values (D, E), respectively. Boxplots represent the medians and standard deviations. Ch: Children; N: Normal; Ad: Adenoma; CRC: Colorectal cancer.

With the analysis of methyl capture sequencing and Illumina BeadChip450K methylation array data, the methylation status of age-related CpG sites and genes was determined during CRC development and progression, and the relevant mRNA expression changes were also evaluated. Among the differentially methylated/expressed age-related genes, *SFRP1* promoter methylation was further analyzed in healthy, premalignant and cancerous colonic tissue samples, including biopsy specimens from young children.

Similarly to previous findings[29], DNA methylation alterations in a considerable proportion of age-related CpG sites/gene promoters (approximately 40%) were observed in samples representing different stages of CRC formation and progression. Approximately two third of these CpG sites had similar DNA methylation alterations in CRC compared to normal tissue samples as during aging. When the effect of DNA methylation of epigenetic clock genes was studied, whole promoter methylation was also observed in addition to the

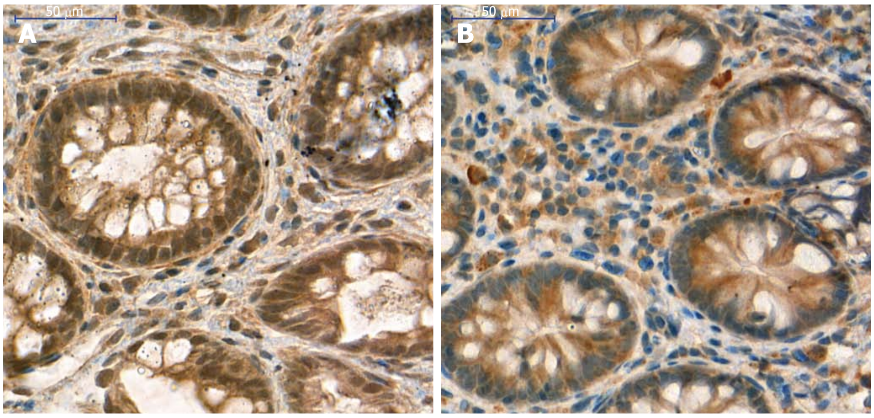

Figure 4 *SFRP1* protein expression in colonic tissue samples of healthy normal children and adults. Strong/moderate cytoplasmic and nuclear *SFRP1* expression both in epithelial and stromal compartments of healthy children (A) and healthy adult (B) samples. Digital microscopic images; magnification × 40; scale: 50 μm.

analysis of DNA methylation status of representing age-related CpG sites.

In accordance with our results, hypermethylation of several genes belonging to aging-associated CpG sites such as *SFRP1*[7,12,15,18-23], *TFAP2E*[30], *TBX5*[31], *GNAS*[32], *DKK3*[18,33], *DGKI*[34], *SYNE1*[35-37], *SDC2*[38,39], *ADHFE1*[40-42] was observed in tissue and/or blood samples of CRC patients. *SFRP1* tumor suppressor protein with a putative Wnt-binding site impedes the frizzled ligand - Wnt receptor interaction. Its reduced expression caused by promoter hypermethylation can lead to constitutive activation of Wnt pathway which is best characterized signaling pathway in CRC pathogenesis[7,12,15,18-23,43]. Worthley *et al*[15] showed strong positive correlation between *SFRP1* methylation and age (Spearmen's rank P = 0.72, P < 0.0001) on a set of 166 CRC tissue samples from adults [median age: 61.2 years (22.8-89.2 years)]. In this study, the increase of *SFRP1* methylation during the aging was also observed, moreover to our best knowledge, we provide the first evidence of significantly lower *SFRP1* methylation levels in children (under 18 years) compared to healthy adult colonic tissues. Preliminary results of methylation-sensitive restriction enzyme methylation array analysis of our reseach groups suggested *SFRP1* hypomethylation in young colonic tissue samples, though the high standard deviations of methylation percentages and the low samples size limited our conclusions[18]. In accordance with the DNA methylation data, elevated mRNA and protein levels could be detected in colonic tissues of normal children compared to adults.

Dickkopf Wnt signaling pathway inhibitors including *DKK3* are also frequent targets of epigenetic silencing in gastrointestinal tumors promoting carcinogenesis by loss of/reduction their expression[33]. Hypermethylated syndecan 2 (*SDC2*) is a potential biomarker for early CRC detection both in serum and tissue, although the gene silencing effect of elevated promoter methylation is not unambiguous according to the literature data[44], moreover some observations support its tumorigenic activity in CRC[45]. Hypermethylation of spectrin repeat containing nuclear envelope protein 1 (*SYNE1*) suggests its tumor suppressor function in CRC[37], which was detected not only in CRC tissue samples[35,36], but it appears to be a promising marker for blood-based CRC detection[37]. Alcohol dehydrogenase, iron containing 1 (*ADHFE1*) promoter hypermethylation was found to be associated with CRC differentiation[41], furthermore it is involved in cell proliferation induction by alcohol in colon carcinoma cells[46].

In the case of some genes like *AKT3*, *CEMIP* and *DDR1*, promoter hypomethylation was observed in different types of cancers such as breast cancer, lung cancer and CRC[47-49]. PI3K/Akt pathway is thought to be the most commonly activated intracellular signaling pathway in human malignancies[50]. AKT kinases including *AKT3* are remarkable contributors to malignant diseases as they are involved in the regulation of cell proliferation, growth and survival[50,51]. Hypomethylation and overexpression of the cell migration-inducing protein (*CEMIP/KIAA1199*) was previously described in CRC[49,52]. In our study, hypomethylation of this gene could be detected mainly in adenoma samples, however a slight but significant decrease in methylation level was observed in CRC samples compared to normal controls. In accordance with the methylation data, strong upregulation of *CEMIP* mRNAs was shown both in adenoma and CRC samples with higher expression values in adenoma tissue[53,54]. Due to its robust overexpression at mRNA and also at protein levels, *CEMIP* is considered as a candidate prognostic marker for CRC and a potential therapeutic target[55]. *CEMIP* facilitates colon cancer cell proliferation *via* enhancing Wnt signaling[49] and promotes tumor growth[55] and cancer dissemination under hypoxic conditions[56].

In conclusion, our results regarding DNA methylation alterations of age-related, epigenetic clock genes during colorectal carcinogenesis supports the concept that aging is one of the main factors predisposing cancer including CRC. Several age-related DNA methylation alterations could be observed during development and progression of CRC affecting the mRNA expression of certain CRC- and adenoma-related key control genes. The main CRC-associated signal transduction pathways, such as WNT signaling and PI3K/Akt pathways are also influenced during aging.

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COMMENTS

Background

Cancer is considered a primary age-related disease, and therefore age-related molecular changes including epigenetic alterations such as epigenetic drift and epigenetic clock necessarily show relationship with carcinogenesis-associated differences. Besides global hypomethylation, local, genomic site specific hypermethylation principally in the promoter regions of tumor suppressor genes can occur during both processes.

Research frontiers

Several age-related DNA methylation alterations could be observed during colorectal cancer (CRC) formation and progression affecting the mRNA expression of certain CRC- and adenoma-related key control genes such as hypermethylated secreted frizzled related protein 1 (*SFRP1*), spectrin repeat containing nuclear envelope protein 1 and hypomethylated cell migration-inducing protein.

Innovations and breakthroughs

For the first time significantly lower *SFRP1* methylation levels were demonstrated in colonic tissue from children (under 18 years) compared to healthy adults. The main CRC-associated signal transduction pathways, such as WNT signaling and PI3K/Akt pathways are also influenced during aging.

Peer-review

In this paper, the authors analyzed the methylation and expression levels of 353 age-related "epigenetic clock" genes in colonic tissue samples. They identified many differentially methylated and/or differentially expressed genes. Among these genes, the DNA methylation and mRNA levels of *SFRP1* was further analyzed. This is an interesting work using a large number of data and the results may be useful in related field.

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