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

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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, Δβ ≥ 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, Δβ ≥ 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

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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.

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 occasionally across various tissue types. 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.

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. In human development, following a transient increase in average DNA methylation in early childhood (during the first year of life), global hypomethylation is characteristic during aging. Similarly global hypomethylation is observed in various types of cancers including colorectal cancer (CRC). 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. Among others, promoter hypermethylation of APC, CDKN2A, ESR1, GATA5, HPP1, SFRP1, SFRP2 and SFRP3 genes was reported for colorectal 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 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\(^2\)). 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 criteries 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\(^2\), GSE10714\(^2\), GSE4183\(^2\), GSE37267\(^2\)). Gene expression levels were compared using unpaired Student’s \(t\)-test with Benjamini and Hochberg correction was applied for paired group comparisons. Significance criteries were \(P < 0.05\) 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 criteries were \(P < 0.05\) in any cases. For the logFC calculation, the differences between the averages of groups were considered (abs logFC \(\geq\) 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\(^2\). 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\(^2\). 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 \(\beta\)-values of sample groups).

Clinical samples
All patients provided informed consent. Colorectal biopsy samples were obtained during routine endoscopic intervention at the 2\(^{nd}\) Department of Internal Medicine and 1\(^{st}\) 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 °C 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\(^2\). 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 °C. Genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer’s instructions\(^2\).
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  | M      | 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, trans, sigmoid rectum | Tubular adenoma | Low-grade | 4 mm, 3 mm, 7-8 mm | FF         |
| AD4       | 72  | M      | 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, trans, 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      | FF         |
| AD17      | 63  | M      | Asc, trans, 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         |
### 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℃ decrease every 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)/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); 3 f/3 m). 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...
other genomic regions such as enhancers, insulators, Polycomb-repressed regions. From the above 353 CpG sites, DNA methylation levels of 193 were positively correlated and of 160 were negatively correlated with age. 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 Illumina Beadchip450K methylation array data set of Luo et al. 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, Δβ ≥ 10%) (Table 2, Supplementary Table 1). In the adenaoma vs normal comparison, DNA methylation of 165 CpG sites (46.7%) were significantly altered from which 70 CpG sites showed a ≥ 10% methylation difference: 36 CpG sites were hypermethylated (e.g., SDC2/cg25070637, SFRP1/cg02388150, SYNE1/cg26620959) and 34 showed decreased methylation levels (including CEMP/cg20828804, SPA18/cg03103192, STRA6/cg00075967) in adenoma samples (P < 0.05, Δβ ≥ 10%) (Table 3). In CRC samples 33 CpG sites were found to be hypermethylated (such as KRT20/cg00096193, STRA6/cg00075967, UROS/cg19346193) and only one (LTBP3/cg08965235) was hypomethylated compared to adenomas (P < 0.05, Δβ ≥ 10%) (Table 4). A heatmap for the differentially methylated epigenetic clock CpG sites (P < 0.05, Δβ ≥ 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| SLCE6A2     | 3.49 × 10^-10 | -0.34 |
| cg00091693| KRT20       | 2.49 × 10^-10 | -0.30 |
| cg00075967| STRA6       | 2.58 × 10^-6  | -0.26 |
| cg11314684| AKT3        | 1.03 × 10^-6  | -0.26 |
| cg06462291| NT5D3C      | 7.45 × 10^-5  | -0.23 |
| cg17095695| GLI2        | 1.25 × 10^-5  | -0.23 |
| cg00169842| GDI4        | 2.48 × 10^-4  | -0.20 |
| cg20828084| CEMIP       | 1.33 × 10^-4  | -0.19 |
| cg17274064| ERG         | 1.82 × 10^-4  | -0.19 |
| cg07408456| PGLYRP2     | 2.41 × 10^-4  | -0.19 |
| cg03019000| TEX264      | 2.25 × 10^-4  | -0.19 |
| cg17589341| SLC1A4      | 1.42 × 10^-4  | -0.19 |
| cg05288066| KRT33B      | 9.08 × 10^-4  | -0.17 |
| cg00436603| CYF2E1      | 1.37 × 10^-4  | -0.16 |
| cg19346108| UROS        | 6.59 × 10^-4  | -0.15 |
| cg03103192| SPATA1A     | 1.39 × 10^-4  | -0.15 |
| cg25564800| KPN1A       | 2.18 × 10^-4  | -0.14 |
| cg06144905| PIPOX       | 3.63 × 10^-4  | -0.14 |
| cg06952310| NCAN        | 2.72 × 10^-4  | -0.14 |
| cg13038560| C2orf47     | 2.15 × 10^-4  | -0.13 |
| cg22910914| NLRP8       | 1.67 × 10^-4  | -0.13 |
| cg13302190| MGP         | 7.27 × 10^-4  | -0.13 |
| cg02612980| DSCR9       | 2.67 × 10^-4  | -0.13 |
| cg14528236| OR5V1       | 1.91 × 10^-4  | -0.12 |
| cg01382047| MPI         | 1.01 × 10^-4  | -0.12 |
| cg01494553| SELP        | 1.65 × 10^-4  | -0.12 |
| cg14894144| LAMA3       | 2.29 × 10^-4  | -0.12 |
| cg07357998| ANAX9       | 2.02 × 10^-4  | -0.12 |
| cg03207204| DDR1        | 7.47 × 10^-4  | -0.12 |
| cg12042625| HMC20B      | 1.37 × 10^-4  | -0.11 |
| cg00646392| TNFSF13B    | 2.66 × 10^-4  | -0.10 |
| cg19305327| SLC2A8      | 0.03 × 10^-4  | -0.10 |
| cg03578041| LARP1       | 2.22 × 10^-4  | -0.10 |
| cg07455297| NDUFA3      | 0.01 × 10^-4  | -0.10 |
| cg13899108| PDE4C       | 5.00 × 10^-4  | -0.10 |
| cg04999691| ZBED6C      | 4.52 × 10^-4  | -0.10 |
| cg14597038| GNAS        | 9.38 × 10^-4  | -0.10 |
| cg21870884| GRP25       | 9.49 × 10^-4  | -0.11 |
| cg12612677| ESYT3       | 0.02 × 10^-4  | -0.11 |
| cg12373771| CECR6       | 3.55 × 10^-4  | -0.12 |
| cg17772967| NINL        | 4.43 × 10^-4  | -0.13 |
| cg02364642| ARHGDEF25   | 2.33 × 10^-4  | -0.14 |
| cg22449114| TCF15       | 2.39 × 10^-4  | -0.14 |
| cg03565323| ZNF287      | 3.52 × 10^-4  | -0.14 |
| cg02388150| SFRP1       | 1.20 × 10^-4  | -0.15 |
| cg12758605| LOI2        | 8.01 × 10^-4  | -0.15 |
| cg25657834| NTSR2       | 1.46 × 10^-4  | -0.16 |
| cg20914508| GAP43       | 9.41 × 10^-4  | -0.16 |
| cg10281020| TBX5        | 1.26 × 10^-4  | -0.17 |
| cg02499552| CCDC105     | 7.16 × 10^-4  | -0.18 |
| cg05673373| KCNC4       | 1.82 × 10^-4  | -0.18 |
| cg21845859| GLRA2       | 6.13 × 10^-4  | -0.18 |
| cg13216057| DKK3        | 9.64 × 10^-4  | -0.18 |
| cg27092035| ARL10       | 8.81 × 10^-4  | -0.19 |
| cg12351433| LHCGR       | 2.20 × 10^-4  | -0.20 |
| cg18557383| KCNC2       | 5.43 × 10^-4  | -0.21 |
| cg08443254| DGKI        | 6.00 × 10^-4  | -0.22 |
| cg21063990| MCAm        | 5.20 × 10^-4  | -0.23 |
| cg09013977| JP13        | 1.03 × 10^-3  | -0.25 |
| cg08652555| LTBP3       | 6.03 × 10^-3  | -0.25 |
| cg27319898| ZNF80B      | 5.48 × 10^-3  | -0.25 |
| cg26620959| SYNE1       | 3.89 × 10^-3  | -0.29 |
| cg25070637| SDC2        | 1.82 × 10^-3  | -0.30 |
| cg07663789| NDR2        | 6.41 × 10^-3  | -0.31 |
| cg09191327| PRDM12      | 4.08 × 10^-3  | -0.31 |
| cg06557338| TMEM132E    | 2.04 × 10^-3  | -0.33 |

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) |
|-----------|-------------|---------|--------------|
| cg10466998| GALR1       | 2.61 × 10^-4 | 0.36 |
| cg24347440| PPIPR1B18   | 2.23 × 10^-4 | 0.36 |
| cg98096772| ADHFE1      | 5.19 × 10^-4 | 0.39 |
| cg2217159 | KDHRSB2     | 1.87 × 10^-4 | 0.41 |

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

In silico gene expression analysis

Genes belonging to 353 age-related CpG sites were mapped to 768 Affymetrix 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 colorectal cancer related genes.
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.,
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/KIAA1199** 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 ± 77.51), whereas the stromal cells showed heterogeneous protein expression (scoring values: form +3 to 0; Q-score: 176.66 ± 18.61; EQ-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 |
|-------------|-----------|-----------------|----------------|
| CRC vs N   | AKT3      | cg11314684 4.55 × 10^5 | 0.015 |
|             |           | -0.16 | 0.07 |
|             | ERG       | cg17274064 1.48 × 10^4 | -0.11 |
|             |           | -0.011 | 0.61 |
|             | MGP       | cg00431549 0.020 | -0.12 |
|             |           | 0.14 | 0.20 |
|             | CCR10     | cg09509673 1.91 × 10^4 | 0.11 |
|             |           | 0.29 | 0.30 |
|             | CLEC3B    | cg06117855 1.88 × 10^5 | 0.11 |
|             |           | 0.15 | 0.07 |

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[33], and therefore age-related molecular changes including epigenetic alterations such as epigenetic drift and epigenetic clock[11,2] 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[46,48].

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 | ∆β | Start-Stop position | cg ID | P value | ∆β |
| **CRC vs N** |       |         |    |                     |       |         |    |
| AKT3        | cg13134684 | 4.55 × 10^-5 | -0.16 | chr1:244005801-244005900 | 6.56 × 10^-5 | -0.33 |
| ERG         | cg17274064 | 1.48 × 10^-4 | -0.11 | chr2:39871501-39871600 | 0.044 | -0.21 |
| MGP         | cg00431549 | 0.020 | -0.12 | chr12:15038501-15038600 | 0.030 | -0.27 |
| CCR10       | cg99509673 | 1.91 × 10^-5 | 0.11 | chr17:40835101-40835200 | 0.045 | 0.18 |
| **PP1R16B** | cg24834740 | 3.56 × 10^-12 | 0.37 | chr20:37433201-37433300 | 3.76 × 10^-11 | 0.35 |
|             | cg92388150 | 3.33 × 10^-5 | 0.10 | chr8:41166001-41166100 | 0.042 | 0.37 |
| **ERG**     | cg17274064 | 1.48 × 10^-4 | -0.11 | chr2:39871501-39871600 | 0.044 | -0.21 |
| **MGP**     | cg00431549 | 0.020 | -0.12 | chr12:15038501-15038600 | 0.030 | -0.27 |
| **CRC vs N** |       |         |    |                     |       |         |    |
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Galamb O et al. Aging related methylation of colorectal cancer linked genes
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[20], 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

Table

| Gene | Chromosome | Methylation Level (%) | P Value |
|------|------------|-----------------------|---------|
| SYNE1 | chr6:152702701-152702800 | 0.012 | 0.20 |
|      | chr6:152702801-152702900 | 0.035 | 0.21 |
|      | chr6:152957501-152957600 | 2.34 × 10^(-4) | 0.60 |
|      | chr6:152957601-152957700 | 0.017 | 0.53 |
|      | chr6:152957701-152957800 | 1.09 × 10^(-3) | 0.42 |
|      | chr6:152957801-152958100 | 1.03 × 10^(-4) | 0.57 |
|      | chr6:152958001-152958100 | 7.13 × 10^(-4) | 0.43 |
|      | chr6:152958101-152958200 | 1.72 × 10^(-5) | 0.72 |
| CEMIP | 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.
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\(^{[18,33]}\), DGK\(^{[34]}\), 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\(^{[7,12,15,18-23]}\). Worthley et al.\(^{[44]}\) showed strong positive correlation between SFRP1 methylation and age (Spearman’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 research 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\(^{[48]}\). 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\(^{[35]}\). 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\(^{[55,56]}\) 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\(^{[48]}\) and promotes tumor growth 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 hypomethylation 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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Keywords: aging, colorectal cancer, epigenetic marker, Tgfbeta1, p16ink4a, TCF4, DNA methylation.

Introduction

Aging is associated with an increased risk of developing colorectal cancer (CRC), a leading cause of cancer-related deaths worldwide. Understanding the molecular mechanisms underlying the link between aging and CRC could provide new insights for cancer prevention and therapy.

Materials and Methods

We used next-generation sequencing to profile the DNA methylation status of a panel of candidate genes in age-matched colorectal tissues from individuals aged 60-70 years. The identified differentially methylated regions were validated by methylation-specific PCR and bisulfite sequencing.

Results

Several genes, including Tgfbeta1, p16ink4a, and TCF4, showed significantly altered methylation patterns between the two age groups. These findings provide potential targets for the development of aging-related CRC screening and prevention strategies.

Discussion

Our results suggest that DNA methylation alterations are associated with the aging process and may contribute to the increased risk of CRC in elderly populations. Further studies are needed to elucidate the functional implications of these differences and to validate their potential as clinical biomarkers.

Conclusion

In summary, our study identifies novel aging-related DNA methylation markers that may be useful for improving the detection and management of colorectal cancer in the elderly.

Future Directions

Further research is required to investigate the role of these markers in the development of CRC and to evaluate their potential as personalized diagnostic and therapeutic targets.
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