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ORIGINAL ARTICLE

Comprehensive DNA Methylation and Mutation Analyses Reveal a Methylation Signature in Colorectal Sessile Serrated Adenomas

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Abstract Colorectal sessile serrated adenomas (SSA) are hypothesized to be precursor lesions of an alternative, serrated pathway of colorectal cancer, abundant in genes with aberrant promoter DNA hypermethylation. In our present pilot study, we explored DNA methylation profiles and examined selected gene mutations in SSA. Biopsy samples from patients undergoing screening colonoscopy were obtained during endoscopic examination. After DNA isolation and quality analysis, SSAs (n = 4) and healthy controls (n = 5) were chosen for further analysis. DNA methylation status of 96 candidate genes was screened by q(RT)PCR using Methyl-Profiler PCR array system. Amplicons for 12 gene mutations were sequenced by GS Junior Instrument using ligated and barcoded adaptors. Analysis of DNA methylation revealed 9 hypermethylated genes in both normal and SSA samples. 12 genes (*CALCA, DKK2, GALR2, OPCML, PCDH10, SFRP1,* *SFRP2, SLIT3, SST, TAC1, VIM, WIF1*) were hypermethylated in all SSAs and 2 additional genes (*BNC1* and *PDLIM4*) were hypermethylated in 3 out of 4 SSAs, but in none of the normal samples. 2 SSAs exhibited *BRAF* mutation and synchronous *MLH1* hypermethylation and were microsatellite instable by immunohistochemical analysis. Our combined mutation and DNA methylation analysis revealed that there is a common DNA methylation signature present in pre-neoplastic SSAs. This study advocates for the use of DNA methylation as a potential biomarker for the detection of SSA; however, further investigation is needed to better characterize the molecular background of these newly recognized colorectal lesions.

Keywords Biomarker · DNA methylation · Mutation · Serrated polyp · Sessile serrated adenoma · Traditional serrated adenoma

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Introduction

Colorectal serrated polyps have become an area of intense focus for gastroenterologists and pathologists over the past several years [1]. Serrated polyps are subdivided into three histological subtypes: sessile serrated adenomas (SSA), traditional serrated adenomas (TSA), and hyperplastic polyps (HP). Whereas the most frequent subtype, HP is regarded as self-limiting and non-neoplastic; SSA and TSA are considered to be pre-neoplastic and hypothesized to be precursor lesions of an alternative pathway leading to colorectal cancer (CRC) [2]. As compared to the classical adenoma-carcinoma pathway driven and characterized by several genetic mutations [3], serrated pathway arises from distinct pre-neoplastic lesions, and it is abundant in genes with aberrant promoter hypermethylation, and termed CpG island methylator phenotype (CIMP) [2]. DNA hypermethylation in the promoter region

can lead through several mechanisms to down-regulation of the affected genes and contribute to colorectal carcinogenesis [4]. Its prominent role in colorectal carcinogenesis has also been utilized as a non-invasive prescreening method for CRC in clinical practice, as it can be detected both in colonic tissue and plasma [5, 6]. SSAs are histologically characterized by a sawtoothed growth pattern, horizontal crypt extensions (inverted T- or L-shaped) at the crypt bases and inverted crypts lying on the mucosal muscle layer (Supplementary Fig. 1) [2]. Patients with SSAs have an increased risk for developing interval CRCs [7, 8] as they are frequently missed by screening colonoscopy due to their flat or sessile morphology and pale color, making them similar to normal colonic mucosa [2]. As colonoscopy is the gold standard method for CRC screening [9], the above observations explain the relatively lower effectiveness of colonoscopy against right-sided CRC (where SSAs tend to be the precursor lesion more commonly) as compared to left-sided CRC [10]. Serrated polyps have also been shown to be poorly detected by fecal occult blood test [11], another important CRC screening method. These observations underline the importance to find alternative methods to improve the detection of serrated polyps, and consequently to optimize CRC screening. Given the key regulatory role of DNA methylation in serrated pathway [2], one can speculate that DNA methylation might be a good biomarker for the early detection of serrated lesions. So far many genes have been examined for DNA hypermethylation in serrated polyps [12–21], but only few studies have focused on multiple genes [22, 23].

The role of genetic and epigenetic alterations in the pathogenesis of serrated adenomas is not fully understood. To further explore this issue, we aimed to analyze promoter DNA methylation status of 96 genes in parallel with 12 frequently described gene mutations (*APC, BRAF, CTNNB1, EGFR, FBXW7, KRAS, MSH6, NRAS, PIK3CA, SMAD2, SMAD4, TP53*) in serrated polyps.

Materials and Methods

Patient Selection and Ethical Considerations

The study was conducted according to the declaration of Helsinki and approved by local ethics committee and government authorities (Regional and Institutional Committee of Science and Research Ethics (TUKEB) Nr.: 69/2008). All subjects provided written informed consent before entering the study, and anonymity was maintained by tracing patients through their clinical history number. Detailed medical history was taken and thorough physical examinations were performed for all participants. The study population encompassed all patients who underwent colonoscopy between January 1, 2011 and December 31, 2012 at the Endoscopy Unit of our Department. Pathological records were searched for "serrated polyps" in the archive of the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University.

DNA Isolation and Sample Selection

DNA was isolated using High Pure PCR Template Preparation Kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions as previously described [24]. As further experiments required high-purity DNA, quality check followed (A260/280 above 1.8 and A260/230 above 1.7) with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Ultimately 4 SSAs, 1 TSA and 5 samples from normal colonic mucosa were chosen for further analysis. Clinical data is shown in Table 1.

DNA Methylation Analysis

DNA methylation of 96 genes were evaluated (Supplementary Table 1) using Methyl-Profiler (EpiTect Methyl qPCR Array System, Qiagen, Hilden, Germany) as described previously [24]. This bisulfite-free DNA methylation analysis is based on the detection of remaining DNA input after cleavage with restriction enzyme digestion followed by quantitative PCR (Supplementary Fig. 2) [25]. Methylation-sensitive restriction enzyme selectively digests the unmethylated DNA, whereas methylation-dependent restriction enzyme cleaves the methylated DNA (Supplementary Fig. 3). Treatment with both enzymes serves as a control for how much DNA is amplified in the assay (Supplementary Fig. 3) [24, 25]. Following enzyme digestion, samples were analyzed by fluorescence-based, quantitative PCR (Supplementary Fig. 2) using LightCycler 480 (Roche) instrument [24]. After the cycling program completed, C_T values were copied into an Microsoft Office Excel (Microsoft, Redmond, WA, USA) file provided

Table 1 Clinical characteristics of analyzed serrated polyps. SSA: sessile serrated adenoma, TSA: traditional serrated adenoma

	SSA 1	SSA 2	SSA 3	SSA 4	TSA
Gender	male	female	male	male	male
Age (years)	59	56	82	78	48
Location	sigmoid	transverse	ascending	ascending	transverse
Size	15 mm	8 mm	5 mm	9 mm	5 mm
Polyp morphology	flat	flat	sessile	sessile	sessile

by the manufacturer, and ΔC_T analysis was performed. The threshold for DNA hypermethylation was set at 15% [24].

Mutation Analysis

Mutation hotspots of 12 frequently described gene mutations in CRC (*APC, BRAF, CTNNB1, EGFR, FBXW7, KRAS, MSH6, NRAS, PIK3CA, SMAD2, SMAD4, TP53*) were amplified by own designed PCR primers [26]. Amplicons were sequenced with a GS Junior Instrument (Roche). In the course of library preparation, rapid Library Molecular Identifier (RL_MID) adaptors were ligated to the PCR products. After ligation, quality of PCR libraries was assessed with the High Sensitivity DNA Chip on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Emulsion PCR amplification of the amplicon libraries was performed using the Lib-L. emPCR Kit (Roche), with 2 DNA molecules per bead ratio following the manufacturer's instructions. GS Junior Titanium Sequencing Kit (Roche) was used for bead enrichment and sequencing steps with the method described in the Sequencing Method Manual, GS FLX Titanium Series. Variants were identified with the Amplicon Variant Analyzer software.

Immunohistochemical Analysis

Immunohistochemical analysis was performed as described previously [24, 27]. Briefly, following dewaxing and rehydration, endogenous peroxidase activity was inhibited with 2.5% hydrogen peroxide in methanol for 30 min, then antigen retrieval was performed in an electric pressure cooker for 40 min in TRIS-EDTA buffer (pH 9). After blocking in 1% bovine serum albumin, slides were incubated overnight at 58 °C with monoclonal antibodies for MLH1 (1:80) (Santa Cruz Biotechnology, Dallas, TX, USA) and SFRP1 (1:150, Abcam, Cambridge, UK). After antibody detection (HISTOLS-MR-T kit, Hisztopatológia Kft, Pécs, Hungary) and visualization (Dako Cytomation, Glostrup, Denmark), slides were counterstained with haematoxylin, dehydrated with xylol, and mounted. After digital archiving (Pannoramic Flash 250 instrument, 3DHISTECH Ltd., Budapest, Hungary), stainings were evaluated with a Pannoramic Viewer digital microscope (software version 1.15; 3DHISTECH) by an experienced researcher (GV).

Results

DNA Methylation Analysis of Serrated Polyps

Analysis of DNA methylation revealed 9 hypermethylated genes (*BAGE, CCNA1, H19, MAGEA1, MGX1, PTGIS, RUNX3, SPARC, UGT1A1*) in both normal and SSA samples (data not shown). 12 genes (*CALCA, DKK2, GALR2,*

OPCML, PCDH10, SFRP1, SFRP2, SLIT3, SST, TAC1, VIM, WIF1) were hypermethylated in all SSAs and 2 additional genes (*BNC1* and *PDLIM4*) were hypermethylated in 3 out of 4 SSAs (Table 2), but in none of the normal samples. Two SSAs (SSA 1 and SSA 2) had hypermethylation in *MLH1*, these samples were also *BRAF* mutant and microsatellite instable (see later). Two additional genes (*APC* and *MGMT*) were hypermethylated in one SSA (SSA 4). TSA showed hypermethylation for only two genes *CALCA* and *SST*, which the only two genes that were hypermethylated in all serrated polyps (Table 2).

Mutation Analysis of Serrated Polyps

Mutation analysis of our samples showed that those two SSAs (SSA 1 and SSA 2), which had also *MLH1* hypermethylation (Table 2) and were microsatellite instable (see later), exhibited only *BRAF* mutation (Table 3). SSA 4, which also had the most hypermethylated genes (Table 2), had three mutations (*APC, FBXW7, TP53*) and SSA 4, similarly to the TSA sample, did not exhibit any mutations (Table 3). Of note, no *KRAS* mutation was detected (Table 3).

Immunohistochemical Analyses of MLH1 and SFRP1

Protein expression for MLH1 was analyzed by immunohistochemistry to assess microsatellite status. Retained MLH1 protein expression (Fig. 1a) translates to proficient mismatch

Table 2 DNA methylation status of serrated polyps

	SSA 1	SSA 2	SSA 3	SSA 4	TSA
APC	um	um	um	**meth**	um
BNC1	**meth**	um	**meth**	**meth**	um
CALCA	**meth**	**meth**	**meth**	**meth**	**meth**
DKK2	**meth**	**meth**	**meth**	**meth**	um
GALR2	**meth**	**meth**	**meth**	**meth**	um
MGMT	um	um	um	**meth**	um
MLH1	**meth**	**meth**	um	um	um
OPCML	**meth**	**meth**	**meth**	**meth**	um
PCDH10	**meth**	**meth**	**meth**	**meth**	um
PDLIM4	um	**meth**	**meth**	**meth**	um
SFRP1	**meth**	**meth**	**meth**	**meth**	um
SFRP2	**meth**	**meth**	**meth**	**meth**	um
SLIT3	**meth**	**meth**	**meth**	**meth**	um
SST	**meth**	**meth**	**meth**	**meth**	**meth**
TAC1	**meth**	**meth**	**meth**	**meth**	um
VIM1	**meth**	**meth**	**meth**	**meth**	um
WIF1	**meth**	**meth**	**meth**	**meth**	um

Bold entries denote pathological changes.

Meth hypermethylated, *SSA* sessile serrated adenoma, *TSA* traditional serrated adenoma, *um* unmethylated

Table 3   Mutation status of serrated polyps

|	SSA 1	SSA 2	SSA 3	SSA 4	TSA
APC	wt	wt	wt	**mut**	wt
BRAF	**mut**	**mut**	wt	wt	wt
CTNNB1	wt	wt	wt	wt	wt
EGFR	wt	wt	wt	wt	wt
FBXW7	wt	wt	wt	**mut**	wt
KRAS	wt	wt	wt	wt	wt
MSH6	wt	wt	wt	wt	wt
NRAS	wt	wt	wt	wt	wt
PIK3CA	wt	wt	wt	wt	wt
SMAD2	wt	wt	wt	wt	wt
SMAD4	wt	wt	wt	wt	wt
TP53	wt	wt	wt	**mut**	wt

Bold entries denote pathological changes.

Mut mutated, *SSA* sessile serrated adenoma, *TSA* traditional serated adenoma, *wt* wild type

repair system and microsatellite stable status, whereas loss of MLH1 protein expression (Fig. 1b) corresponds to deficient mismatch repair system and microsatellite instable status. In SSA 1 and SSA 2, that also exhibited *MLH1* methylation (Table 2) and *BRAF* mutation (Table 3), MLH1 protein was absent (Fig. 1b), so these samples were declared microsatellite instable. The remaining samples (SSA3, SSA4, TSA and the normal samples) retained MLH1 staining (Fig. 1a), and were considered to be microsatellite stable.

SFRP1, one of the genes that were hypermethylated in all SSA samples, was chosen to be analyzed at protein level by immunohistochemistry. Accordingly, SFRP1 protein expression seen in normal colonic mucosa samples (Fig. 1c) was reduced in SSA samples (Fig. 1d).
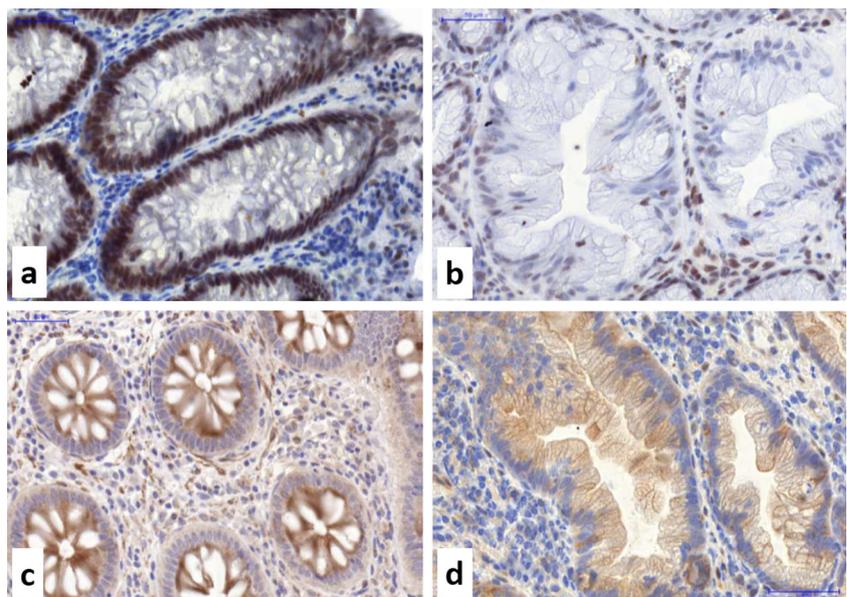
Discussion

Our combined mutation and methylation analysis showed that there is a common DNA methylation signature present in pre-neoplastic SSAs as opposed to TSA and normal controls. These findings are in line with our previous study [24], where we showed that pre-neoplastic lesions and CRCs arising in the classical adenoma-carcinoma pathway have a characteristic DNA methylation profile, as well. Interestingly, 6 genes (*BNC1, SFRP1, SFRP2, SLIT3, SST, WIF1*) were found to be frequently hypermethylated not only in CRC, but in both pre-neoplastic traditional adenomas and sessile serrated lesions, that warrants further investigation with larger sample size. As serrated polyps have recently been shown to be poorly detected by fecal occult blood test [11], these hypermethylated genes could be exploited as novel stool or blood biomarkers for the non-invasive detection of these polyps.

Previous studies examining DNA methylation in serrated polyps have either analyzed a single gene [16, 18, 20, 21] or a few well-described (usually CIMP) genes [12–15, 17, 19, 22, 23]. Compared to these previous works, we managed to identify some additional candidate hypermethylated genes.

Majority of earlier studies evaluated *BRAF* and *KRAS* mutation status, as *BRAF* mutation was found to be a characteristic for SSA and *KRAS* mutation for TSA [14], although the specificity and the sensitivity is very low. In addition to *KRAS* and *BRAF*, we analyzed 10 other mutations that had been frequently described in the classical adenoma-carcinoma pathway. Although due to the small sample size we cannot draw firm conclusions from our results, it seems that these examined additional genetic mutations are scarce in serrated carcinogenesis, but this should be further examined in subsequent studies.

Fig. 1 Nuclear MLH1 protein expression characteristic of normal colonic epithelial cells (**a**) is absent in an SSA sample (**b**). SFRP1 protein expression detected in normal epithelial cells (**c**) is decreased in SSA (**d**). Magnification: 40×, scale bar: 50 μm. SSA: sessile serrated adenoma

Similarly to earlier observations, we found that *MLH1* hypermethylation leads to loss of MLH1 expression at protein level. It was recently shown that promoter methylation and loss of protein expression are not parallel processes, and *MLH1* hypermethylation is an earlier molecular event that occurs also in HP, whereas loss of protein expression is a much later step [28].

There are several limitations to our study. Samples were collected in a single center, and only pre-neoplastic serrated polyps were included. The major drawback of our study is its relatively small sample size. However, in contrast to earlier molecular studies on serrated polyps where mostly only a single or a few genes were examined, we followed a different approach and analyzed the genetic and epigenetic alterations of more than 100 genes. It is also highly debatable, that we included only a single TSA into our study, however as it was shown in a recent, large cohort study from the Netherlands [29], TSA is an extremely rare type of serrated polyps, even in internationally acknowledged, expert centers. In this well-described cohort, among 744 serrated polyps, only one lesion was diagnosed as a TSA, comprising 0.1% of all colorectal polyps. During our study period 2 TSAs were identified; however, the other TSA was excluded from the study during the preanalytical phase due to inappropriate quality of the isolated DNA. We still decided to include a single TSA because of its vastly different mutation and methylation profile, and to stimulate further molecular studies to support or disprove our observations on this enigmatic polyp.

In conclusion, we demonstrated that in line with previous results, DNA methylation is a frequent event in SSAs and several novel genes have been described for the first time. We hope that our results can provide data for subsequent studies and inspire further research on this topic, including genome-wide screening of DNA methylation [30] in serrated polyps. This would provide important insights into the pathogenesis of serrated polyps and subsequent cancers, and also yield potential new biomarkers that could be used for the better detection of these lesions, which could ultimately lead to improved CRC screening and better management of this common, but preventable disease.

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