Serrated polyps

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IJspeert, J.E.G.

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CHAPTER 2

Clinical, histological and molecular features of sessile serrated polyps caught in the act of malignant progression

J.E.G. IJspeert, R.J. Reinten, S. van Eeden, E. Dekker, C.J.M. van Noesel

ABSTRACT

Background

The (epi)genetic alterations responsible for the malignant progression in sessile serrated polyps (SSP) are only marginally understood. We aimed to evaluate which cancer pathways are associated with early progression of SSP and to compare features of MLH1 deficient and MLH1 proficient SSP with a focus of dysplasia or cancer.

Methods

All SSP with a focus of dysplasia or cancer of < 10 mm, diagnosed from 2006 onwards in the Academic Medical Centre were centrally revised. Eligible lesions were included for analysis. Sections were immunostained for β-catenin, p53, SMAD4 and MLH1. DNA was extracted from the non-progressed and the progressed components of lesions and examined for CpG Island methylator phenotype (CIMP) status, microsatellite instability (MSI) and the presence of mutations within a panel of 23 genes by next generation sequencing. The sequenced reads were used to assess the degree of single nucleotide variation within MSI and microsatellite stable (MSS) lesions respectively.

Results

In total 35 SSP with a focus of dysplasia or cancer were included. Progressed components more often showed loss of MLH1 (60% vs 0%; p<0.001), evidence of WNT pathway activation (17% vs 0%; p=0.04), TP53 dysfunction (29% vs 0%; p<0.01) and TGF-β pathway dysfunction (23% vs 0%), as compared to non-progressed components. A BRAF mutation (present in 97% of both components) and CIMP-high phenotype (86% vs 71%; p=0.06) were equally often found in both components. Loss of MLH1 within the progressed component was associated with female gender (90% vs 57%; p=0.02), diagnosis at older age (median 68 vs 58 years; p<0.01) and MSI (100% vs 0%; p<0.001), while inversely associated with WNT pathway activation (5% vs 36%; p=0.02) and TGF-β pathway dysfunction (10% vs 43%; p=0.02). MSI and MSS lesions demonstrated a similar degree of single nucleotide variations (p=0.51), while a non-significant trend was seen for MSI carcinomas, as compared to MSI dysplasia.

Conclusion

The clinical and molecular profiles of SSP with early progression critically depend on the MLH1 expression status. As compared to MLH1 deficient lesions, proficient lesions are more often driven by WNT activation or functional impairment of the TGF-β pathway and more often found in male patients of younger age. MSI can be found even in the smallest lesions with loss of MLH1 expression, but only seems to result in the initiation of a hypermutated profile in SSP that have progressed to cancer.

BACKGROUND

For decades, tubular and (tubulo-)villous adenomas were considered the only type of polyps with malignant potential, progressing to colorectal cancer (CRC) via the so called canonical adenoma-carcinoma pathway.[1,2] However, more recent molecular studies have shown that 15-30% of all CRCs arise from sessile serrated polyps (SSP).[3,4] Evaluation of the molecular characteristics of serrated polyps and a subcategory of CRCs has provided insight in the pathogenesis of the serrated neoplasia pathway.[5,6] Generally, a mutation in the BRAF oncogene is the first step in this sequence, potentially resulting in enhanced proliferation and/or reduced apoptosis followed by cell senescence in the absence of additional (epi)genetic alterations. [7,8] Silencing of tumor suppressor genes, e.g. due to CpG island methylation of promoter regions, enables cells to bypass the senescence barrier.[7–9] In particular, methylation and subsequent silencing of the mismatch repair gene MLH1 is a defining event as it causes overt genetic instability and progression to BRAF mutant/microsatellite-instable (MSI) cancer.[10] A minority of SSP progress with proficient mismatch repair as a result of other (epi)genetic alterations, such as a TP53 mutation and/or loss of P16, resulting in BRAF mutant/microsatellite-stable (MSS) cancer.[10,11]

Although the serrated neoplasia pathway is broadly explored, the genetic and epigenetic alterations primarily responsible for malignant progression of SSP are only marginally understood. One of the main reasons is the fact that SSP with dysplasia are genetically highly instable and supposed to quickly progress to full blown cancer.[10,12] Consequently, SSP with a focus of dysplasia or cancer are rare and only seldom resected, impeding options for molecular studies.[13] Secondly, due to the impaired function of the DNA repair system, full-blown MSI cancers demonstrate a hypermutated phenotype with hundreds to thousands of nonsynonymous mutations.[14–18] As a result, true driver gene mutations and passenger mutations are very difficult to differentiate, hindering a proper reconstruction of key genetic alterations accompanying cancer initiation and tumor growth in the serrated neoplasia pathway. Molecular evaluation of the earliest forms of progressed SSP (with a focus of dysplasia or carcinoma) would help to overcome these issues and could reveal the (epi)genetic alterations accompanying the malignant transition in SSP.

We have studied a comprehensive panel of SSP with a focus of dysplasia or cancer. The aim of this study was to evaluate which cancer cell signaling pathways are associated with progression in SSP and to characterize and compare the clinico-histopathological as well as molecular features of MLH1 deficient and MLH1 proficient SSP with a focus of dysplasia or carcinoma.

METHODS

Study design and case selection

This study represents a cohort of all SSP with a focus of either dysplasia or cancer diagnosed from 2006 onwards within the pathology unit of the Academic Medical Centre in Amsterdam, the Netherlands. All lesions that were initially diagnosed as SSP with a component of progression (dysplasia or cancer) were centrally revised by two expert GI-pathologists (CvN and SvE). Lesions were included If all of the following criteria were met; A) presence of both a component of non-progressed SSP as well as a component of either dysplasia or carcinoma; B) presence of a clear and abrupt transition from non-progressed SSP to dysplasia or cancer within the same tissue sample; C) absence of any features of a traditional serrated adenoma; D) presence of a component of dysplasia or cancer of ≤10mm. Criteria A-C were defined to ensure the inclusion of only unequivocal SSP with dysplasia or cancer. Criterion D) was defined to ensure inclusion of progressed lesions in the earliest stage of disease. The study was conducted in accordance with the research code of the medico-ethical institutional review board of the Academic Medical Center and was performed in agreement with the Helsinki Declaration.[19]

Clinico-pathological data collection

For each lesion patient as well as polyp characteristics were retrieved from the electronic medical charts. Included patient characteristics were age at diagnosis, gender, history of CRC, total number of detected adenomas as well as SSP and a diagnosis of serrated polyposis syndrome. Polyp characteristics were also collected: size and location, as reported in the endoscopy report. Rate of progression (low grade dysplasia (LGD), high grade dysplasia (HGD) or cancer) was reviewed during a joined central polyp revision by CvN and SvE. Lesions were assessed according to the World Health Organization Classification of Tumours of the digestive system.[20]

IHC analysis

Immunohistochemical (IHC) analysis was performed as earlier described.[21] Unstained 5-µm slides were cut from paraffin blocks and deparaffinized. Slides were submerged for 20 minutes in 0.3% hydrogen peroxide in methanol. Slides were boiled for 20 minutes in a solution of 10 mmol/L Tris and 1 mmol/L EDTA (pH 9) and incubated for one hour at room temperature with one of the selected primary antibodies. The primary monoclonal antibodies used were specific for MLH1 (1:50; BD Pharmingen, San Diego, CA); SMAD4 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA); β-catenin (1:10.000; BD Biosciences, San Diego, CA), and p53 (1:2000; Neomarkers Inc., Fremont, CA). After incubation, antibodies were blocked (ImmunoLogic, Duiven, the Netherlands) in PBS and implemented by an antipolyvalent horseradish peroxidase detection system (ImmunoLogic) to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin. Sections stained for β-cateninwere regarded positive for aberrant WNT pathway activation when strong nuclear staining was observed in at least 25% of the cells. Sections stained for p53 were considered as indicative of TP53 dysfunction if at least 75% of the lesional nuclei were strongly

positive or completely negative. Sections stained for SMAD4 were considered as indicative for TGF-β pathway dysfunction in case of a complete absence of nuclear staining in all lesional cells. Similarly, sections stained for MLH1 were considered indicative for MLH1 loss in case of a complete absence of nuclear staining in all lesional cells. Lesional cells of the non-progressed SSP component and the progressed component were evaluated separately.

DNA Isolation

Formalin-fixed paraffin-embedded tissue sections were used to micro-dissect lesional cells from the non-progressed SSP component, the progressed component and from normal tissue separately. DNA was extracted using proteinase K digestion.

CIMP analysis

CpG Island methylator phenotype (CIMP) status of normal cells, non-progressed SSP cells and progressed SSP cells was assessed using the SALSA MLPA CIMP kit (MRC Holland, Amsterdam, the Netherlands) according to manufacturer's instruction. This kit covers methylation markers for IGF2, SOCS1, NEUROG1, RUNX3, CACNA1G, MLH1, CRABP1 and CDKN2A (P16) and evaluates the presence of a V600e mutation in the BRAF oncogene. The level of methylation was calculated using the Coffalyser software (MRC Holland, Amsterdam, the Netherlands). Lesions were considered CIMP-high if at least 5/8 CIMP markers were methylated.

MSI analysis

Microsatellite status of all the transformed components of the SSP was determined using the MSI Analysis System v1.2 (Promega, Madison, USA), which makes use of a standard panel of five microsatellite markers (NR-21, NR-24, MONO-27, BAT25, and BAT26). Analyses were performed according to manufacturer's instruction. MSI-high was defined as at least two (40%) unstable markers, MSI-low as one unstable marker, and MSS as no unstable markers.

Next generation sequencing

Next generation sequencing was performed using the Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 (ThermoFisher Scientific, Waltham, Massachusetts, USA) for targeted multi-gene amplification (14,6 kb) according to manufacturer's instruction. This panel exists of hotspots of the following genes; KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBX7, FGFR3, NOTCH1, ERBB4, FGFR1 and FGFR2. Libraries were prepared using the ION PGM Hi-Q OT2 Kit and Ion OneTouch-2 Instrument were used for emulsion PCR and template preparation. Finally, the Ion PGM Hi-Q sequencing Kit with the Ion 318 V2 Chip and Personal Genome Machine were used as sequencing platform. DNA input was up to 20 ng, which was measured by the Qubit 3.0 Fluorometer. Up to 20 specimens were barcoded using the IonXpress Barcode Adapters for each Ion 318 V2 Chip. A background noise of 5% was chosen to determine the total number of existing non-synonymous driver-gene mutations per lesion. A background noise of

1% was chosen to determine the single nucleotide variation within each lesion. These data were used to quantify and compare nucleotide instability within MLH1 deficient and MLH1 proficient lesions. Of note, the selected sequencing panel was developed to identify most mutations within the included genes. However, not all exones of each included gene were analyzed, harboring a risk of undetected mutations in tumor suppressor genes.

Statistical analysis

Descriptive statistics were presented as means with standard deviation for normally distributed continuous data, as median with either range or interquartile range for non-normally distributed continuous data and as percentage for categorical data. Chi-square test, Fisher exact test, McNemar test and Mann-Whitney U test were used to compare groups. SPSS statistics (version 23; SPSS, Chicago, Illinois, USA) was used for statistical analyses. A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics

In total 35 SSP with a focus of dysplasia or cancer of ≤10 mm were included for analysis (Table 1). Median age at diagnosis of an SSP with dysplasia or cancer was 64 years (IQR 59-71) and eight (23%) lesions were detected in male patients. In total, 15 (43%) patients had a history of CRC and 19 (54%) patients fulfilled the 2010 WHO criteria for serrated polyposis syndrome. The median number of diagnosed adenomas within the patient cohort was two (IQR 1-6), and the median number of SSP was six (IQR 2-8).

In total 17 (48%) lesions were diagnosed as SSP with LGD, nine (26%) as SSP with HGD and nine (26%) as SSP with CRC. The median size of included lesions was 10 mm (IQR 8-16) and the median size of the progressed component was 4 mm (IQR 2-5mm). In total 31 (89%) lesions were located in the proximal colon.

Non-progressed vs progressed SSP component

In Table 2 the molecular characteristics of the non-progressed and progressed component of included SSP are presented. IHC analysis demonstrated loss of MLH1 within the progressed component of 21 (60%) lesions, which was not present in the non-progressed polyp component in any of these lesions ($p<0.001$). Accordingly, IHC showed nuclear β-catenin staining as evidence of WNT pathway activation in 17% of progressed vs 0% of non-progressed components ($p=0.04$), TP53 dysfunction in 29% of progressed vs 0% of non-progressed components ($p<0.01$) and lack of SMAD4 expression reflecting TGF-β pathway dysfunction in 23% of progressed vs 0% of non-progressed components ($p=0.01$).

Table 1 | Patient and polyp characteristics

	Overall (n=35)	MLH1 deficient lesions (n=21)	MLH1 proficient lesions (n=14)	p-value
Patient characteristics				
Age in years; median (IQR)	64 (59-71)	68 (63-73)	58 (49-64)	<0.01
Male gender; n (%)	8 (23)	2 (10)	6 (43)	0.02
History of CRC; n (%)	15 (43)	9 (43)	6 (43)	1
No. of diagnosed adenomas; median (IQR)*	2 (1-6)	2 (1-5)	2 (1-10)	0.73
No. of diagnosed SSP; median (IQR)*	6 (2-8)	6 (4-11)	3 (2-6)	0.04
Diagnosed with SPS; n (%)	19 (54)	14 (67)	5 (36)	0.07
Polyp characteristics				
Size in mm; median (IQR)	10 (8-16)	10 (8-18)	13 (8-16)	0.74
Size progressed component in mm; median (IQR)	4 (2-5)	4 (2-5)	4 (2-5)	0.59
Proximal location; n (%)	31 (89)	20 (95)	11 (79)	0.13
Type of progression; (n %)				0.35
LGD	17 (48)	10 (48)	7 (50)	
HGD	9 (26)	7 (33)	2 (14)	
CRC	9 (26)	4 (19)	5 (36)	

* Cumulative number of all lesions detected at diagnosis and during surveillance
CRC = colorectal cancer; SPS = serrated polyposis syndrome; SSP = sessile serrated polyp; LGD = low-grade dysplasia; HGD = high-grade dysplasia

DNA analysis demonstrated that a pathogenic V600E BRAF mutation was present in 34 (97%) of the included lesions, both in the non-progressed as well as the progressed component. A CIMP high phenotype was found in the non-progressed and progressed components of 25 (71%) and 30 (86%) lesions respectively (p=0.06). Most markers already showed methylation in a high percentage before transition to dysplasia or cancer (RUNX3, NEUROG1, IGF2, CRABP1, CACNA1G). In accordance with IHC analysis, MLH1 methylation was solely demonstrated in the progressed component of SSP lacking MLH1 protein expression. Methylation of CDKN2A (P16) was found in the non-progressed component of 18 (51%) SSP and in the progressed component of 26 (74%) SSP (p=0.01). Methylation of SOCS1 was not found in any of the evaluated lesions. Cells from normal colon crypts adjacent to the 35 included SSP were evaluated as negative control. None of the normal colon crypt cells showed a BRAF mutation or methylation of any of the eight included CIMP-markers.

MLH1 deficient vs MLH1 proficient lesions

In Table 1, the clinico-pathological characteristics of 21 progressed SSP with loss of MLH1 (MLH1 deficient lesions) are presented, as compared with 14 progressed SSP with MLH1 expression (MLH1 proficient lesions), as assessed by IHC. No significant difference was found between groups in progression rate, total size of the lesion or size of the progressed component, suggesting comparable groups with regard to stage of disease. MLH1 proficient SSP with dysplasia or cancer occurred at a

Table 2 | Molecular comparison of non-progressed and progressed component of 35 sessile serrated polyps

	Non-progressed component	Progressed component	p-value
IHC analysis; n (%)			
MLH1 loss	0 (0)	21 (60)	<0.001
Activated WNT pathway	0 (0)	6 (17)	0.04
Dysfunctional P53 pathway	0 (0)	10 (29)	<0.01
Dysfunctional TGF-β pathway	0 (0)	8 (23)	0.01
DNA analysis; n (%)			
BRAF mutation	34 (97)	34 (97)	1
CIMP-high	25 (71)	30 (86)	0.06
*MLH1 proficient lesions**	7 (50)	9 (64)	0.50
*MLH1 deficient lesions***	18 (86)	21 (100)	0.25
MLH1 methylation	0 (0)	22 (63)	<0.001
RUNX3 methylation	33 (94)	33 (94)	1
NEUROG1 methylation	34 (97)	34 (97)	1
CDKN2A methylation	18 (51)	26 (74)	0.01
IGF2 methylation	32 (91)	31 (89)	1
CRABP1 methylation	28 (80)	33 (94)	0.13
SOCS1 methylation	0 (0)	0 (0)	1
CACNA1G methylation	26 (74)	29 (83)	0.38

* Sub-analysis in SSP with proficient function MLH1 in the progressed component
** Sub-analysis in SSP with loss of function MLH1 in the progressed component

significantly younger age, as compared to MLH1 deficient SSP with dysplasia or cancer (median 68 years vs 58 years; p<0.01) and were more often detected in male patients (43% vs 10%; p=0.02). A non-significant trend was seen for the association between the diagnosis of serrated polyposis syndrome and a MLH1 deficient phenotype (p=0.07), while significantly more SSP were diagnosed in patients with a MLH1 deficient lesion as compared to patients with a MLH1 proficient lesion (p=0.04).

In Table 3, the molecular characteristics of the progressed component of MLH1 deficient and MLH1 proficient SSP are presented. As assessed by IHC analysis, MLH1 proficient lesions more often demonstrated activation of the WNT pathway (36% vs 5% of lesions; p=0.02) and alterations in the TGF-β pathway (43% vs 10% of lesions; p=0.02). No statistical difference was found for TP53 dysfunction, although results show a trend for more frequent dysfunction in MLH1 proficient lesions (43% of lesions vs 19% of lesions; p=0.13).

MLH1 deficient lesions more often demonstrated a CIMP-high phenotype as compared to MLH1 proficient lesions (100% vs 64% of lesions; p<0.01). Comparable results were found, whether or not methylation of MLH1 was taken into account for the calculation of a CIMP-high phenotype. In addition to MLH1, also CDKN2A (P16) was more often methylated in MLH1 deficient lesions (90% vs 50% of lesions; p=0.02).

Table 3 | Molecular comparison of the progressed component of MLH1 deficient and MLH1 proficient sessile serrated polyps

	MLH1 deficient lesions (n=21)	MLH1 proficient lesions (n=14)	p-value
IHC analysis; n (%)			
Activated WNT pathway	1 (5)	5 (36)	0.02
Dysfunctional P53 pathway	4 (19)	6 (43)	0.13
Dysfunctional TGF-β pathway	2 (10)	6 (43)	0.02
DNA analysis; n (%)			
CIMP-high	21 (100)	9 (64)	<0.01
MSI	21 (100)	0 (0)	<0.001
TP53 mutation	4 (19)	4 (29)	0.51
FBXW7 mutation	3 (14)	0 (0)	0.15
FGFR2 mutation	2 (10)	0 (0)	0.23
No. pathogenic driver-gene mutations; median (range)	1 (1-4)	1 (0-3)	0.78

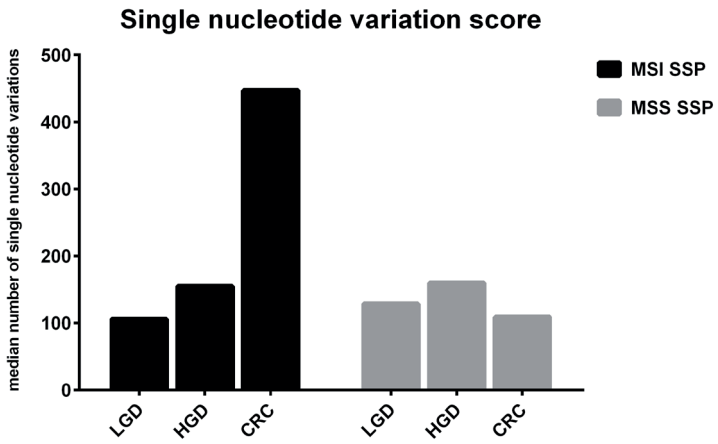

Figure 1 | Median number of clonal and subclonal (threshold ≥1% of cells) single nucleotide variations in sessile serrated polyps with low-grade dysplasia, high-grade dysplasia and cancer, as detected with Ion AmpliSeq™ Colon and Lung Cancer Research Panel. Data were stratified for the presence of microsatellite instability.

All 21 (100%) MLH1 deficient lesions demonstrated MSI, compared to none of the MLH1 proficient lesions (p<0.001). Next generation sequencing demonstrated a comparable number of mutations within the MLH1 proficient lesions (median 1; range 0-3) and the MLH1 deficient lesions (median 1; range 1-4)(p=0.78). Mutations that were found in at least two lesions are presented in Table 3. A TP53 mutation was found in eight (23%) lesions, a FBXW7 mutation in three (9%) lesions and a FGFR2 mutation in two lesions (6%). Mutations in CTNNB1, ERBB2, PTEN and DDR2 were found in one (3%)

lesion. No statistical difference was found between MLH1 deficient and MLH1 proficient lesions in the occurrence of specific mutations. With regard to the degree of nucleotide variation among the sequence reads of the various amplicons, MLH1 proficient lesions demonstrated a median number of 133 (IQR 92-278) single nucleotide variations that exceeded a background level of 1%, while MLH1 deficient lesions demonstrated a median number of 145 (IQR 111-290) single nucleotide variations (p=0.51). For both subgroups no correlation was found between size of dysplasia or cancer and number of detected single nucleotide variations. Figure 1 demonstrates the median number of single nucleotide variations for the progressed component of MSS and MSI SSP, stratified for stage of progression. Statistical analysis was not performed due to cohort size. In Figure 2 the clinical and molecular characteristics of all 35 individual SSP containing dysplasia or cancer are presented, as stratified by immunohistochemical MLH1 expression.

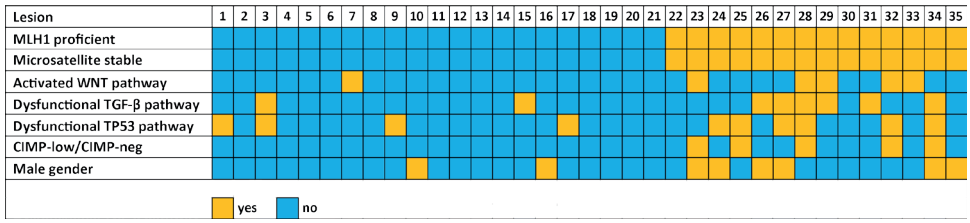

Figure 2 | Clinical and molecular characteristics of progressed sessile serrated polyps stratified by MLH1 expression. Immunohistochemically, lesions 1-21 showed no MLH1 expression and lesions 22-35 showed MLH1 expression.

DISCUSSION

Up to date, the molecular alterations directly accountable for the malignant transformation of SSP are only marginally understood. To gain insight in this process, we studied the molecular alterations in the earliest stages of SSP progression. We noticed two separate pathways of carcinogenesis in SSP, both characterized by unique molecular as well as clinical characteristics. In the first pathway (demonstrated in 60% of cases), lesions showed an early BRAF mutation, broad promoter CpG island methylation, loss of MLH1 and subsequent MSI. Those lesions were mainly found in women of older age. In the second pathway (demonstrated in 40% of cases), lesions showed an early BRAF mutation, mild promoter CpG island methylation, without affecting the MLH1 gene, but with dysfunctional WNT, TP53 and/or TGF-β pathways. As compared to MLH1 deficient lesions, these lesions were less often found in women (90% vs 57%; p=0.02) and more often diagnosed in patients of younger age (median 68 vs 58 years; p<0.01).

Results from the current study should be evaluated in the context of recent efforts to characterise CRC subgroups based on molecular characteristics. To facilitate uniform practice, a large international

consortium has recently developed an overarching classification system, identifying four CRC molecular subtypes based on gene expression profiles.[22] In this classification system consensus molecular subtype 1 (CMS1), characterized by MSI, frequent BRAF mutations and a hypermutated as well as CIMP-high phenotype, shows remarkable comparison with our group of MLH1 deficient SSP with dysplasia or cancer. Results from the current study suggest that the clinical and molecular profile of CMS1 precursor lesions is rather homogeneous. We demonstrated that loss of MLH1 results in microsatellite instability, even in the most diminutive lesions. For that reason, loss of MLH1 alone seems sufficient to initiate a rapid course of carcinogenesis in SSP. Inactivation of the mismatch repair system results in disturbed repair of replication errors of tandem repeat sequences made, genome-wide, by DNA polymerases. The ensuing insertions and deletions when present in protein-coding regions cause reading frame shifts and subsequent inactivation of cancer-related tumor suppressor genes such as TGFBR2, MSH3 and BAX.[23] This process eventually results in a hypermutated phenotype, as seen in MSI CRC.[14,15,18] However, in our study we demonstrated a similar number of single nucleotide variations between MSI and MSS lesions. Therefore, an initial stage of a hypermutated profile in progressed SSP with MSI could not be substantiated. This could potentially be due to the fact that other DNA repair mechanisms, such as the base-excision repair system, partially adopt the function of the mismatch repair system, diminishing the DNA damage. Alternatively, simply more cell divisions and more extensive genomic analyses are needed to quantify the hypermutated profile. Interestingly, a comparison stratified for stage of progression suggests that the DNA repair mechanism is still functional in the dysplastic component of MSI SSP, while compromised in malignant MSI SSP. As shown in Figure 1, MSS SSP with dysplasia or cancer and MSI SSP with dysplasia harbour a comparable number of single nucleotide variations. However, this number increased dramatically in MSI SSP with a component of cancer. Unfortunately, due to the small sample size this observation could not be statistically validated.

In addition to the above mentioned lesions, SSP with dysplasia or cancer and a MLH1 proficient phenotype show similarities with BRAF mutated CMS4 CRC.[22] Since especially cancers that are BRAF mutated and MSS possess an unfavourable prognosis, understanding of the carcinogenesis of these lesions seems of great importance.[24,25] Results from our study suggest that the molecular profile of these lesions is more heterogeneous than that of MLH1 deficient progressed SSP, with a variable quantity of CpG island methylation and dysfunction in different cell signalling pathways within individual lesions. Specific frequently mutated genes were not found using next generation sequencing. These results, together with the fact that these SSP were found in patients of younger age, suggest that methylation of MLH1 is a relatively late epigenetic phenomenon, that only occurs in the absence of other major genomic alterations, such as dysfunction of the WNT, TP53 and/or TGF-β pathway.

Several other studies assessed the clinical and molecular characteristics of SSP with dysplasia or cancer, which could be compared with results from the current study. Recently, the clinical and

molecular characteristics of 137 SSP with a component of dysplasia or cancer were presented.[10] In this study loss of MLH1 was found in 75% of SSP with dysplasia or cancer, which is higher than shown in the current study (60%). In comparison, studies that assessed the molecular profile of full-blown CRC, demonstrated MLH1 methylation in 60-67% of CIMP-high/BRAF mutated cancers.[3,14] This is probably caused by random sampling error and should not be seen as a true difference in prevalence of disease. In agreement with our study, distinct clinico-pathological subgroups were identified, based on loss of MLH1.[10] As compared to MLH1 proficient SSP, MLH1 deficient SSP were more often found in women (70% versus 36%; p<0.0008) of older age (76.7 versus 71.0; p<0.0029). A finding that was also demonstrated in full-blown MSI vs MSS cancers that developed via the serrated neoplasia pathway.[14,26,27] Furthermore, loss of MLH1 was associated with a proximal location (91% versus 72%; p<0.02), CIMP-high phenotype (98% versus 80%; p<0.02) and lack of aberrant p53 (7% versus 34%; p<0.001). In contrast to our study, nuclear β-catenin (dysfunction in WNT pathway) was often found in MLH1 deficient as well as MLH1 proficient SSP with dysplasia or CRC (54% vs 56%), and already described in the non-progressed SSP component of 11% of lesions. This argues with findings from our study and the results from an earlier report, demonstrating that sporadic MSI CRC rarely display immunohistochemical evidence of WNT pathway activation.[28] Furthermore, CDKN2A (P16) silencing was associated with malignant progression, irrespective of loss of MLH1.[10] However, in our study methylation of CDKN2A (P16) was significantly more often found in MLH1 deficient lesions (90% vs 50% of lesions; p=0.02). Furthermore, CDKN2A (P16) methylation was also already seen in 51% of non-progressed SSP components. Most interestingly, in the Bettington study, lesions with dysplasia were found at a similar age as compared to lesions harbouring a carcinoma. Furthermore, CRC was also already found in small and diminutive lesions, as also described in the current study as well as in earlier reports.[29,30] This might suggest a very rapid progression from SSP to dysplasia to CRC, after a relatively long dwell time of SSP. The relatively low proportion of detected SSP with dysplasia, in comparison to non-progressed SSP and CIMP-high/BRAF mutant CRC further strengthens this hypothesis.[3,13,14,31] Unfortunately, in the study by Bettington et al. DNA was not isolated from the non-progressed and progressed component of each lesion, hindering comparison with our analyses.

In the current study, we used very strict in- and exclusion criteria, and evaluated only SSP with an unequivocal diagnosis. Therefore, collision with other polyp subtypes, such as adenomas and TSA was excluded. Furthermore, as far as we are aware, this was the first study to isolate and compare the DNA of both the non-progressed and progressed component of SSP, building on an earlier study from our group.[21] To enable more extensive analysis in future studies, cohort size should be enhanced, also increasing the number of SSP with a component of CRC. Future studies should focus on whole exome sequencing as well as whole methylome analyses to obtain better insight in the oncogenes and tumor suppressor genes involved in the earliest steps of carcinogenesis within SSP. Expectedly, such analyses will unveil the molecular mechanisms underlying the hypermutative state of MSI CRC.[14–17,26]

In conclusion, we demonstrated two independent pathways accompanying malignant progression in SSP. The first pathway, characterized by an early BRAF mutation, broad promoter CpG island methylation, loss of MLH1 and subsequent MSI was present in 60% of cases and almost exclusively detected in SSP in the proximal colon of women at a relatively older age. Interestingly, MSI did not directly result in a hypermutated profile, as seen in full-blown MSI CRC. The second pathway, characterized by an early BRAF mutation, a milder promoter CpG island methylation phenotype and frequent dysfunctional signalling in the WNT, TP53 and/or the TGF-β pathway was present in 40% of lesions, both in male and female patients of a relatively younger age. Identification of these pathways in SSP "caught in the act" to become cancer contributes to understand early carcinogenesis of either BRAF mutated/MSI (pathway one) as well as BRAF mutated/MSS (pathway two) CRC and could eventually enhance current options for screening and treatment.

REFERENCES

1. Muto T, et al. The evolution of cancer of the colon and rectum. Cancer 1975;6:2251–70.
2. Fearon EF, et al. A genetic model for Colorectal Tumorigenesis. Cell 1990;61:759–67.
3. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:113–30.
4. Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.
5. Fernando WC, et al. The CIMP phenotype in BRAF mutant serrated polyps from a prospective colonoscopy patient cohort. Gastroenterol Res Pract 2014;article in press.
6. Burnett-Hartman AN, et al. Genomic aberrations occurring in subsets of serrated colorectal lesions but not conventional adenomas. Cancer Res. 2013;73:2863–72.
7. Bettington M, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013;62:367–86.
8. IJspeert JEG, et al. Serrated neoplasia-role in colorectal carcinogenesis and clinical implications. Nat Rev Gastroenterol Hepatol 2015;12:401–9.
9. Carragher LAS, et al. V600EBraf induces gastrointestinal crypt senescence and promotes tumour progression through enhanced CpG methylation of p16INK4a. EMBO Mol Med 2010;2:458–71.
10. Bettington M, et al. Clinicopathological and molecular features of sessile serrated adenomas with dysplasia or carcinoma. Gut 2015; article in press.
11. Kriegl L, et al. Up and downregulation of p16(Ink4a) expression in BRAF-mutated polyps/adenomas indicates a senescence barrier in the serrated route to colon cancer. Mod Pathol 2011;24:1015–22.
12. Sheridan TB, et al. Sessile serrated adenomas with low- and high-grade dysplasia and early carcinomas: an immunohistochemical study of serrated lesions 'caught in the act'. Am J Clin Pathol 2006;126:564–71.
13. Abdeljawad K, et al. Sessile serrated polyp prevalence determined by a colonoscopist with a high lesion detection rate and an experienced pathologist. Gastrointest Endosc 2015;81:517–24.
14. The Cancer Genome Atlas. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–7.
15. Timmermann B, et al. Somatic mutation profiles of MSI and MSS colorectal cancer identified by whole exome next generation sequencing and bioinformatics analysis. PLoS One 2010;5:1–10.
16. Donehower LA, et al. MLH1 -silenced and non-silenced subgroups of hypermutated colorectal carcinomas have distinct mutational landscapes. J Pathol 2013;229:99–110.
17. Lin EI, et al. Mutational profiling of colorectal cancers with microsatellite instability. Oncotarget 2015;6:42334–44.
18. Seshagiri S, et al. Recurrent R-spondin fusions in colon cancer. Nature 2012;488:660–4.
19. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 1997;310:2191–4.
20. Snover DC, et al. Serrated polyps of the colon and rectum and serrated polyposis. In: Bosman T, Carneiro F, Hruban R, et al. WHO classification of tumours of the digestive system. Lyon, 2010:160–5.
21. Boparai KS, et al. A serrated colorectal cancer pathway predominates over the classic WNT pathway in patients with hyperplastic polyposis syndrome. Am J Pathol 2011;178:2700–7.
22. Guinney J, et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350–6.
23. Kim TM, et al. The Landscape of Microsatellite Instability in Colorectal and Endometrial Cancer Genomes. Cell 2013;155:858–68.
24. Samowitz WS, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005;65:6063–9.
25. De Sousa E Melo F, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat Med 2013;19:614–8.
26. Kim JH, et al. Distinct features between MLH1-methylated and unmethylated colorectal carcinomas with the CpG island methylator phenotype: implications in the serrated neoplasia pathway. Oncotarget 2016;7:14095–111.
27. Lash RH, et al. Sessile serrated adenomas: prevalence of dysplasia and carcinoma in 2139 patients. J Clin Pathol 2010;63:681–6.
28. Panarelli NC, et al. Sporadic microsatellite instability-high colon cancers rarely display immunohistochemical evidence of Wnt signaling activation. Am J Surg Pathol 2015;39:313–7.

29. Bouwens MWE, et al. Endoscopic characterization of sessile serrated adenomas/polyps with and without dysplasia. Endoscopy 2014;46:225–35.
30. Rosty C, et al. Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management. J Gastroenterol 2013;48:287–302.
31. IJspeert JEG, et al. Prevalence, distribution and risk of sessile serrated adenomas/polyps at a center with a high adenoma detection rate and experienced pathologists. Endoscopy 2016; article in press