The serrated neoplasia pathway: investigating the role of serrated polyps in colorectal cancer development

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A serrated colorectal cancer pathway predominates over the classical WNT-pathway in patients with hyperplastic polyposis syndrome

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ABSTRACT

Background and aims:
Hyperplastic polyposis syndrome (HPS) is characterized by the presence of multiple colorectal serrated polyps and is associated with an increased colorectal cancer (CRC) risk. The mixture of distinct precursor lesion types and malignancies in HPS provides a unique model to study the canonical pathway and a proposed serrated CRC-pathway in humans.

Methods:
To establish which CRC-pathways play a role in HPS and particularly to obtain new support for the serrated CRC pathway, we assessed the molecular characteristics of polyps (n=84) and CRCs (n=19) in 17 HPS patients as compared to control groups of various sporadic polyps (n=59) and sporadic, microsatellite-stable CRCs (n=16).

Results:
In both HPS and sporadic polyps, APC mutations were exclusively identified in adenomas whereas BRAF mutations were confined to serrated polyps. Six of 19 (32%) HPS CRCs were identified within a serrated polyp. Mutation analysis performed in both the CRC and the serrated component of these lesions showed identical BRAF mutations. One HPS CRC was located within an adenoma, both components harboring an identical APC mutation. Overall, 10/19 (53%) HPS CRCs carried a BRAF mutation, compared to none in control group CRCs (p=0.001). Six (60%) of the BRAF-mutated HPS CRCs were microsatellite-unstable (MSI-high) due to MLH1 methylation.

Conclusion:
Our findings provide novel supporting evidence for the existence of a predominant serrated CRC pathway in HPS, generating both microsatellite-stable and microsatellite-instable CRCs.
INTRODUCTION

Colorectal cancer (CRC) ranks as the second most common cause of cancer-related death in the western world.1 The classical model which describes CRC development is the adenoma-carcinoma sequence associated with activation of the WNT signalling pathway.2,3 This pathway is characterised by an initial, bi-allelic inactivation of the adenomatous polyposis coli gene (APC) followed by mutations in key oncogenes and tumor-suppressor genes, including KRAS, DCC and TP53, resulting in adenoma initiation and progression to CRC. This multi-step process of carcinogenesis has been elaborately studied and much information has been derived from the familial adenomatous polyposis (FAP) and MUTYH-associated polyposis (MAP) syndromes.

In addition to the adenoma-carcinoma sequence, an alternative, microsatellite instability (MSI) pathway exists which is characterized by deletion or inactivation of mismatch repair (MMR) genes. Loss of one of the MMR genes occurs in 10-15% of the sporadic CRC, whereas 1-2% of CRCs with MMR gene loss is due to a hereditary predisposition, i.e. in Lynch syndrome patients carrying a mono-allelic MMR gene defect in the germline.

Recently a, “serrated neoplasia pathway”, has been proposed which involves the progression of serrated polyps, i.e. hyperplastic polyps (HPs), sessile serrated adenomas (SSAs) and/or traditional serrated adenomas (TSAs), to CRC. The early genetic events of this route, as yet identified, are BRAF or KRAS mutations and an enhanced CPG-island methylation status of multiple genes.4-11 There is evidence to suggest that a proportion of sporadic MSI CRCs originate from serrated polyps as both these lesions commonly harbour hypermethylated MLH1 in combination with BRAF mutations.12-16 In addition, clinicohistological reports supporting a serrated CRC pathway
include CRCs in close vicinity of large hyperplastic polyps\textsuperscript{17, 18}; CRCs identified in mixed hyperplastic and adenomatous polyps\textsuperscript{19}; and increased incidence of serrated polyps in patients with sporadic microsatellite-unstable CRCs.\textsuperscript{4, 10, 20} Currently however, proof of the existence of a serrated CRC pathway, demonstrated by the combined histological finding of a serrated polyp directly adjacent to a CRC and concurrent molecular evidence for a sequential relationship has not been delivered.

Hyperplastic Polyposis Syndrome (HPS) is a condition characterized by the presence of multiple colorectal serrated polyps. The genetic cause(s) of HPS is/are largely unknown. We recently demonstrated that HPS can occur in the context of MAP\textsuperscript{21}, but \textit{MUTYH} mutations seem to occur in only a small proportion of HPS patients.\textsuperscript{22} HPS is associated with an increased CRC-risk.\textsuperscript{5, 7, 19, 22-27} Previously published case series report CRC at clinical presentation in up to 50\% of HPS patients and interval carcinomas, i.e. carcinomas occurring after HPS diagnosis and during endoscopic surveillance, in up to 25\% of patients.\textsuperscript{24, 28} However, since HPS is a heterogeneous condition, comprising serrated polyps of different categories i.e. HPs and SSAs but also co-existent conventional adenomas\textsuperscript{22, 23, 28-30}, it is uncertain which polyps eventually lead to CRC in these patients and thus are clinically relevant. In the case that the CRCs do originate from the serrated polyps, HPS may prove to be a valuable model for studying the serrated CRC pathway.

Within a cohort of 56 HPS patients, we identified 17 patients with CRC. By combined histopathological and molecular analyses of the polyps and CRCs in these patients, we obtained novel evidence for a serrated CRC pathway in HPS which predominates over the classical \textit{WNT} pathway of carcinogenesis.
MATERIALS AND METHODS

Subjects
From a cohort of 56 HPS patients undergoing endoscopic treatment/surveillance at the Academic Medical Centre in the Netherlands, 21 HPS patients had CRC. From 17 of these patients tissue was available (n=19) which was included in this study. HPS was defined as at least five histologically diagnosed HPs and/or SSAs proximal to the sigmoid colon, of which 2 greater than 10mm in diameter, or more than 20 HPs and/or SSAs distributed throughout the colon. Because both HPs and SSAs are common findings in HPS and have been shown to be difficult to differentiate microscopically, all serrated polyps were included in our criteria. Patients with a known germline APC mutation or a bi-allelic MUTYH mutation were excluded from the study. The study was conducted in accordance with the research code of our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983.

Specimens
All retrieved CRCs (n=19) were formalin-fixed and paraffin-embedded. H&E stained tissue sections were re-evaluated by two pathologists (CvN, SvE). In addition, all CRCs were re-evaluated for the presence of an adjacent serrated component and for features of a serrated adenocarcinoma as claimed by others. A control group was selected consisting of sporadic, microsatellite stable (MSS) CRCs (n=14) from non-polyposis patients, matched for age, gender and CRC-location. These CRCs were re-evaluated as described above.

In the case of a mutation identified in a CRC, ≥ 5 polyps in the closest proximity of the CRC were selected and reviewed by a single
pathologist (CvN) who was blinded for patient characteristics and original histological diagnosis. Polyps were classified as HP, SSA, TSA, mixed polyp or conventional adenoma based on the histological features on H&E staining.\textsuperscript{35-37} Polyps with a serrated morphology i.e. HPs, SSAs, TSAs and mixed polyps were collectively designated as ‘serrated polyps’. A polyp control group was also selected consisting of sporadic HPs (n=24), SSAs (n=18) and conventional adenomas (n=17) from non-polyposis patients. For the purpose of analysis, lesions from the caecum, ascending colon, transverse colon and descending colon were regarded as proximal and those from the sigmoid colon and rectum were regarded as distal.

\textit{Somatic mutation analysis}

Epithelial cells from polyps and CRCs were microdissected and DNA was isolated as described previously.\textsuperscript{38, 39} Using previously described primers and assays, DNA was analysed for mutations in the \textit{APC}-mutation cluster region (\textit{APC}-MCR), \textit{KRAS} (exon 2), \textit{BRAF} (exon 15) and \textit{NRAS} (exons 1 and 2).\textsuperscript{38, 39} In the case of a CRC in a polyp, mutation analysis of \textit{TP53} (exons 4-10) was performed in an attempt to assess whether these two components were clonally related. In case of an identified genetic mutation in a CRC, mutation analysis of surrounding polyps (≥5) was performed as described above. Detected mutations were confirmed in a second independent experiment.

\textit{Microsatellite instability analysis}

Microsatellite status of the CRCs of HPS patients was determined using an international standard panel of 5 microsatellite markers (D17S250, D2S123, D5S346, BAT25 and BAT26) using standard techniques. A high degree of microsatellite instability (MSI-high) was
 defined as two (40%) or more unstable markers, MSI-Low as one unstable marker, and microsatellite stable (MSS) as no unstable markers.

*Immunohistochemistry*

Immunohistochemistry was performed on CRCs and polyps of the HPS patients. Unstained 5-µm sections were cut from paraffin blocks and the slides were deparaffinized. Primary monoclonal antibodies used were specific for MLH1(1:50 BD Pharmingen, San Diego, USA); MSH2(1:100 Oncogene Research Prod., San Diego, USA); MSH6(1:200 BD Transduction Lab., San Jose, USA); PMS2(1:250 BD Transduction Lab., San Jose, USA); SMAD4(1:200 Santa Cruz, USA); CTNNB1(1:10.000 BD Biosciences, San Diego, USA) and TP53(1:2000 Neomarkers, Fremont, USA). Slides were immersed in 0.3% hydrogen peroxide in methanol for 20 minutes. Subsequently, antigen retrieval was carried out by 10 minutes of boiling in 10mM Tris/1mM EDTA (pH 9) followed by incubation with above mentioned diluted primary antibodies during 1 hour at room temperature. Post-antibody block (Immunologic) in PBS was performed followed by implementation of an antipolyvalent HRP detection system (Immunologic) to visualize antibody binding sites with 3.3'-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

Immunoreactivity for CTNNB1 (β-catenin) was regarded as positive when strong nuclear staining was observed in >25% of the cells. Stains for TP53 were regarded to be indicative of TP53 dysfunction or deletion when >75% of the lesional nuclei were strongly positive or completely negative (absent staining). Stains for
SMAD4, MLH1, MSH2, MSH6 and PMS2 were considered negative when there was complete absence of nuclear expression in all lesional cells. Negative staining in a part of a lesion or in a single crypt was registered separately.

**Statistics**

Statistical analyses were performed by using a statistical software package (Statistical Package for the Social Sciences 12.0.2; SPSS Inc, Chicago, Ill). Somatic mutations in CRCs and polyps of HPS patients were compared with those of a control panel using a two-sided Fisher exact test. A p-value of < 0.05 was considered statistically significant.

**RESULTS**

**Patients**

The clinico-pathological features of the HPS patients are summarized in table 1. The median age of this cohort of 17 HPS patients at CRC diagnosis was 58 years (range: 41-75) with a male: female ratio of 8:9. In all patients, germline APC and MUTYH-mutation analyses were previously performed and found negative. A surgical colonic resection was performed in 14/17 (82%) patients: 6 subtotal colectomies, 4 hemi-colectomies (2 right-sided) and 4 (recto)sigmoidal resections. Histological evaluation of biopsies and surgical resection specimens of these patients revealed a median of 16 HPs, 7 SSAs and 2 conventional adenomas per patient. All patients satisfied the criteria for HPS defined by the World Health Organization (WHO).
**Mutation analysis in HPS polyps and control-group polyps**

A total of 84 polyps, originating from 17 HPS patients with CRC, were analysed for pathogenic mutations in the *APC*-mutation cluster region (*APC*-MCR), *KRAS* (codons 12 and 13), *BRAF* (codon 600) and *NRAS* (exons 1 and 2). The 84 HPS polyps consisted of 21 HPs, 38 SSAs, 3 TSAs, 2 mixed polyps (64 serrated polyps) and 20 conventional adenomas. The control group of sporadic lesions (n=59) consisted of 24 HPs, 18 SSAs (42 serrated polyps) and 17 conventional adenomas (table 2).

Molecular analysis, of both the HPS polyps and control group polyps, showed *APC*-MCR mutations exclusively in the conventional adenomas and *BRAF* mutations exclusively in the serrated polyps: *BRAF* mutations were detected in 48/64 (75%) HPS serrated polyps, whereas 20/42 (48%) of the control group serrated polyps harbored a *BRAF* mutation (p=0.007). Also when evaluating HPS patients individually, each patient harbored predominantly *BRAF* mutations in their serrated polyps. In four patients, beside *BRAF* mutations a single *KRAS* mutation was identified in a distal serrated polyp. No significant difference in frequency of *KRAS* mutations in serrated polyps was seen between groups (6% vs 17%; p=0.1), but in conventional adenomas of HPS patients no *KRAS* mutations were detected, compared to 4/17 (24%) conventional adenomas in the control group (p=0.029). In none of the polyps *KRAS* and *BRAF* mutations were found together.
Table 1. Clinico-pathological features of HPS patients with colorectal cancer
G= gender, Location=location of carcinoma, AC=ascending colon, TC= transverse colon DC=descending colon

When location in the colon was analyzed, no association was seen between the presence of a **BRAF** mutation and polyp location in both HPS and in the control group (table 3). **BRAF** mutations were observed in 36/46 (82%) proximal HPS serrated polyps and in 12/18 (67%) distal HPS serrated polyps. In HPS, **KRAS** mutated serrated
polyps (4/15: 27%) were exclusively detected in the distal colon (p=0.005).

Immunohistochemistry in polyps
In none of the 84 HPS polyps, loss of expression of the mismatch repair genes (MLH1, MSH2, MSH6 and PMS2) or SMAD4 was observed (not shown). In addition, none of these polyps showed abnormal TP53 staining (either strong nuclear TP53 staining or complete TP53 loss, not shown). In 9 conventional HPS adenomas, we detected strong nuclear CTNNB1 staining (i.e. in >25% of lesional cells, not shown). In 5 of these adenomas, an APC-MCR mutation was identified. In four other APC-MCR mutated adenomas no abnormal nuclear β-catenin staining was detected. Nuclear CTNNB1 was not found in any of the serrated polyps of either group.

<table>
<thead>
<tr>
<th>Somatic mutation</th>
<th>Patients with HPS</th>
<th>Control group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serrated polyps (n=64)</td>
<td>AD (n=20)</td>
<td>Serrated polyps (n=42)</td>
</tr>
<tr>
<td>APC mutation</td>
<td>0</td>
<td>9 (45%)</td>
<td>0</td>
</tr>
<tr>
<td>BRAF mutation</td>
<td>48 (75%)</td>
<td>0</td>
<td>20 (48%)</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>4 (6%)</td>
<td>0</td>
<td>7 (17%)</td>
</tr>
</tbody>
</table>

Table 2. Detected APC, KRAS and BRAF mutations in serrated polyps (HPs, SSAs, TSAs and mixed polyps) and adenomas (AD) compared to a control panel.
*Statistically significant p-value for BRAF mutation frequency in serrated polyps of HPS patients compared to serrated polyps in the control group.
**Statistically significant p-value KRAS mutation frequency in adenomas of HPS patients compared to adenomas in the control group.
**Histological characteristics of the colorectal carcinomas**

Upon histological re-evaluation of CRCs, 6/19 (32%) HPS CRCs were identified within/directly adjacent to a serrated polyp. In 1/19 (5%) cases, the HPS CRC was identified within a conventional adenoma (Fig.1). One patient had two synchronous CRCs. Although 6 CRCs were identified within serrated polyps, no HPS CRCs displayed a distinguishing morphology justifying the diagnosis serrated adenocarcinoma.\textsuperscript{10}

<table>
<thead>
<tr>
<th>Mutation:</th>
<th>(\text{BRAF} \text{ mutation} )</th>
<th>(\text{KRAS} \text{ mutation} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group:</td>
<td>HPS</td>
<td>Control group</td>
</tr>
<tr>
<td>Location:</td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td>HP</td>
<td>8/13 (62)</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>SSA</td>
<td>25/30 (83)</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>TSA</td>
<td>1/1 (100)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>MP</td>
<td>2/2 (100)</td>
<td>0/0</td>
</tr>
</tbody>
</table>

**Table 3.** Serrated polyp location and \(\text{BRAF} \) and \(\text{KRAS} \) mutation. MP=mixed polyp

NOTE. All values are expressed as n (%).
Mutation analysis in HPS CRCs and control-group CRCs

*BRAF* mutations were detected in 10/19 (53%) HPS CRCs, whereas no *BRAF* mutations were detected in the CRCs of the control group (p=0.001, table 4 and figure 2). All *BRAF* mutations involved the same thymine to adenine transversion at nucleotide 1796, resulting in a valine to glutamine substitution at codon 600 (V600E). We found no
NRAS mutations in the CRCs of either group. Of the 6 CRCs identified within a serrated polyp, 5/6 (83%) carried the same BRAF mutation (GTG → GAG) in both the polypous and tumor components (Table 5). However, considering that these are hotspot mutations, these findings do not prove a clonal relationship between these lesions. TP53 (exons 4-10) sequence analysis, performed on these 6 CRCs in order to further explore the clonal relationship between the carcinomas and precursor lesions, yielded no mutations.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>HPS carcinomas (n=19)</th>
<th>Carcinomas control group (n=14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>2 (11%)</td>
<td>4 (29%)</td>
<td>Ns</td>
</tr>
<tr>
<td>KRAS</td>
<td>1 (5%)</td>
<td>5 (36%)</td>
<td>Ns</td>
</tr>
<tr>
<td>BRAF</td>
<td>10 (53%)</td>
<td>0</td>
<td>0.001*</td>
</tr>
<tr>
<td>NRAS</td>
<td>0</td>
<td>0</td>
<td>Ns</td>
</tr>
<tr>
<td>MSS</td>
<td>12 (63%)</td>
<td>14</td>
<td>NA</td>
</tr>
<tr>
<td>MSI-L</td>
<td>1 (5%)</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>MSI-H</td>
<td>6 (32%)</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 4. Mutation spectrum of colorectal cancers in HPS patients compared to control-group CRCs. ns: not significant; NA: not applicable. *statistically significant p-value compared to control group CRC
APC-MCR mutations were identified in 2/19 (11%) HPS CRCs compared to 4/14 (29%) in the control group CRCs (not significant) and 1/19 (5%) KRAS mutations were detected in HPS CRCs compared to 5 (36%) in the control group (not significant). In one HPS CRC (patient 6), both a BRAF mutation and an APC-MCR mutation were detected. Interestingly, one of the HPS CRCs (patient 11), harbouring an APC-MCR mutation (insertion 1364), was the single HPS CRC found within a villous adenoma (Fig.1C). Subsequent APC-MCR sequence analysis of the adenoma revealed the same mutation (insertion 1364), establishing a clonal relationship between these
lesions. Accordingly, both the adenomatous and malignant components displayed evident nuclear CTNNB1 staining (Fig. 3).

When location in the colon was analysed, all BRAF-mutated HPS CRCs were exclusively detected in the proximal colon (p=0.007). No association was seen between location and KRAS mutations in the control group or APC-mutations in both groups. The entire list of molecular analyses on a per-carcinoma basis can be found as a supplementary table online (http://ajp.amjpathol.org).

<table>
<thead>
<tr>
<th>Carcinoma</th>
<th>Adjacent polyp</th>
<th>Mutation in CRC</th>
<th>Mutation in adjacent polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>HP</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>SSA</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
</tr>
<tr>
<td>8</td>
<td>HP</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
</tr>
<tr>
<td>10</td>
<td>SSA</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
</tr>
<tr>
<td>11</td>
<td>Adenoma</td>
<td><strong>APC</strong> (Insertion G 1354)</td>
<td><strong>APC</strong> (Insertion G 1354)</td>
</tr>
<tr>
<td>15</td>
<td>SSA</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
</tr>
<tr>
<td>16</td>
<td>SSA</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
<td><strong>BRAF</strong> (GTG→GAG)</td>
</tr>
</tbody>
</table>

**Table 5.** Mutations in CRCs and adjacent polyps.
Genetic events detected immunohistochemically and by microsatellite analysis in CRCs

In 6/19 (32%) HPS CRCs, loss of expression was seen of at least 1 mismatch repair (MMR) protein. In 6 CRCs, there was complete loss of expression of both MLH1 and PMS2. All these 6 HPS CRCs were microsatellite-unstable (MSI-high), were proximally located and harboured a \textit{BRAF} mutation. Two of the 6 MSI-high CRCs involved combined serrated polyp-CRC lesions. In these lesions, the serrated polyp components were microsatellite stable (MSS).

Strong nuclear $\beta$-catenin staining ($>25\%$) was observed in 5/19 (26%) HPS CRCs. In one of these CRCs, an \textit{APC-MCR} mutation was identified and none of these was \textit{BRAF} mutated. In all 5 of these HPS CRCs, abnormal TP53 staining was observed ($>75\%$ nuclear staining or complete absence), indicative of loss of functional TP53. Three additional HPS CRCs displayed a perturbed TP53 status. In 2/19 (11%) CRCs loss of SMAD4, either focal or complete, was detected.
DISCUSSION

In this first comprehensive cohort study of HPS patients with both CRCs and polyps, we demonstrated that the serrated polyps and conventional adenomas of HPS patients not only morphologically resemble their respective sporadic counterparts but also have similar molecular profiles (table 3, figure 2). Interestingly, we identified a high number of combined serrated polyp-CRC lesions which showed identical \textit{BRAF} mutations in both components, supporting the existence of a serrated CRC pathway. Overall, we demonstrated that both microsatellite-stable and –unstable CRCs in HPS predominantly originate from the serrated polyps thus confirming that HPS patients provide a valuable model to analyze the molecular characteristics of the serrated CRC pathway.

In accordance with previous reports \cite{13, 40, 41}, the HPS serrated polyps harboured significantly more \textit{BRAF} mutations than those of the control group (80 vs. 48\%: \( p=0.001 \)). The slight difference in the \textit{KRAS} mutation frequencies between both groups (7 vs. 17\%) was not statistically significant. Thus in HPS patients, \textit{BRAF} mutations correlate even stronger with serrated polyps than in non-HPS patients. This contrasts with serrated polyps in MAP patients with a germline \textit{MUTYH} gene mutation, which contain significantly more \textit{KRAS} mutations (70\%) than \textit{BRAF} mutations (4\%).\cite{21} Both \textit{BRAF} and \textit{KRAS} have been identified as early or instigating events in the serrated pathway. It has to be determined however, whether serrated lesions with these mutually exclusive mutations are biologically equivalent and have the same risk of developing to CRC.

The mutation profiles of the HPS CRCs were clearly more similar to those found in the serrated polyps than in the conventional adenomas (table 3 and figure 2). The profiles of the control group MSS
CRCs, as expected, were comparable to the profiles of the conventional adenomas. As many as 10 of the included 19 HPS CRCs were \textit{BRAF}-mutated compared to none of the 14 control group CRCs (p=0.001). Interestingly, all these \textit{BRAF}-mutated CRCs were proximally located. Previous molecular studies in HPS CRCs encompassed small non-controlled series of selected cases.\textsuperscript{23, 42-44} and a comparison between our results and these studies is not straightforward owing to variation in patient selection criteria and analysis of different or single genes. Overall however, \textit{BRAF} mutations were identified in 6/15 (40%) HPS-related CRC-cases described in the assembled literature until now. We identified no \textit{BRAF} mutations in the control group of age-and sex-matched, MSS CRCs from non-polyposis patients. Concordantly, \textit{BRAF} mutations have been reported in only 5-10\% of sporadic MSS CRCs of non-polyposis patients (n>100).\textsuperscript{45-48}

Considering that in the literature serrated polyps are suggested to be precursor lesions of particularly MSI CRCs, we chose MSS carcinomas in our control group in order to exclude MSI-CRCs from potentially unrecognized HPS patients. However, this strategy is arbitrary and eliminates sporadic MSI CRCs from non-HPS patients. Hence, our statistically significant difference in \textit{BRAF} mutation frequency between HPS CRCs and control group CRCs is higher compared to an unselected cohort of CRCs. We detected only one \textit{KRAS} mutation in a distally located HPS CRC, which corroborates two previously published studies together analyzing a total of 15 HPS CRCs.\textsuperscript{40, 41} To our knowledge, only one other study has described the presence of a \textit{KRAS} mutation in a single distally located HPS CRC, supporting the notion that \textit{KRAS} plays a minor role in the carcinogenesis of HPS and is confined to the distal colon.\textsuperscript{23}
In our study, 2 patients had synchronous CRCs. Theoretically, a field effect may account for the development of synchronous polyps/CRCs. In one patient (patient 9), no mutations were identified in both CRCs. The other patient (patient 17) harboured one KRAS mutation and one BRAF mutation respectively in each of the CRCs. Hence, although a field effect associated with a serrated CRC pathway may cause simultaneous CRCs with identical mutations, we were not able to demonstrate this in patient 9 due to the lack of any mutations and seems excluded in patient 17. Of note, clonal markers are necessary to be able to demonstrate a true field effect. Such clonal markers associated with the serrated pathway are currently not available.

The histological characteristics of the HPS CRCs as a group, either or not BRAF-mutated, were inconspicuous and not obviously different from the control group CRCs. In particular, a serrated growth pattern, as has been reported, was not apparent in our series of HPS CRCs. We found a remarkable number of HPS CRCs (6/19: 32%) to be located within or directly adjacent to a serrated polyp (Fig. 1). In 5/6 of these combined lesions an identical hotspot mutation at codon 600 (GTG→GAG) of BRAF was detected in both components. These combined histological and molecular data are strongly suggestive for a sequential relationship between serrated polyps and CRC. A clonal relationship between the carcinomas and their assumed precursor lesions could not be further substantiated due to lack of appropriate clonal markers.

In the literature it has been shown that 90% of sporadic MSI-H CRCs are caused by loss of MLH1 function as a result of methylation of this mismatch repair gene. In these lesions, BRAF mutations, which are associated with serrated polyps, are also a common finding
suggesting that the serrated pathway can generate MSI-H CRCs.\textsuperscript{13, 15} Interestingly, 6/19 HPS CRCs were MSI-H due to loss of \textit{MLH1} and \textit{PMS2} and all 6 carried \textit{BRAF} mutations. Additional analysis in these CRCs also showed hypermethylation of MLH1 in all cases (data not shown). These findings suggest a causal relationship between this novel carcinogenic route and microsatellite-unstable CRCs. On the other hand, our findings demonstrate that the serrated pathway, at least in HPS, also generates MSS-CRCs.

Previous large cohort studies (>30 patients) have reported the presence of at least one conventional adenoma in 69- 85\% of HPS patients and >5 conventional adenomas in 21-32\% of cases.\textsuperscript{22, 23, 28-30} Concordantly, in our study \textit{APC-MCR} mutations were detected in two (13\%) HPS CRCs. The identification of an HPS CRC (patient 11) located within a conventional adenoma of the same clonal origin, reflected by the identical \textit{APC-MCR} mutation in both components, is significant since this proves that the classical adenoma-carcinoma pathway\textsuperscript{52} is also operational in HPS patients.

The apparent dominance of the serrated over the non-serrated CRC pathway in HPS may either be due to a greater intrinsic risk of tumour progression of the serrated polyps, or be simply a reflection of the numerical prevalence of serrated polyps over adenomas. To our knowledge, the only study that has addressed this issue in 2 HPS CRC cases, reported no \textit{APC} mutations.\textsuperscript{23} In that study however, patients with >10 conventional adenomas were excluded. In our study, all cases satisfied the WHO criteria for HPS in which the presence or number of conventional adenomas is not an issue. Interestingly, in HPS patient 11 with the classical CRC, a relative abundance of conventional adenomas was observed (12 adenomas versus 17 serrated polyps), suggesting a stochastic rather than an intrinsically
biased process of carcinogenesis. Interestingly, in the other APC-MCR-mutated HPS CRC (patient 6) also a BRAF mutation was identified. Assuming that this CRC is of monoclonal origin, it may be the outcome of an elusive ‘fusion’ pathway which combines mechanisms associated with both conventional adenomas and serrated polyps, as proposed previously.\textsuperscript{36}

We observed 4 HPS CRCs with nuclear CTNNB1 staining, associated with the WNT-pathway. Although in these CRCs, no APC-MCR mutations or CTNNB1 exon 4-10 hotspot mutations were identified, alternative mechanisms may be operational to activate the WNT-pathway, e.g. methylation of the APC promotor regions and MSI-related frameshift mutations in WNT pathway regulators like AXIN2.\textsuperscript{53} In these HPS patients, a median of only 1 adenoma (range: 0-5) was identified, suggesting that the WNT-pathway indeed may be involved at some stage of carcinogenesis via the serrated pathway. Considering that no BRAF mutations or directly adjacent serrated polyps were found, it seems unlikely that these CRCs are the outcome of a proposed fusion pathway, but formally this can not be excluded.

Based on these observations, we conclude that distinct, APC- and non-APC mediated CRC pathways are functional in HPS. A serrated pathway, skewed towards initial BRAF mutations and proximal localization, however seems to predominate, most likely due to the numerical prevalence of serrated polyps in these patients. From this it is inferred that all polyp types in HPS, should be considered clinically relevant and be removed.
REFERENCES


Chapter 6


148


