Research Article

Hyperplastic Polyps Are Innocuous Lesions in Hereditary Nonpolyposis Colorectal Cancers

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Aims. To compare methylation profiles, protein expression, and microsatellite instability (MSI) of sporadic, HNPCC, and familial hyperplastic polyps (HPs). Methods. Methylation-specific PCR (MSP) and pyrosequencing assessed p16, MGMT, hMLH-1, MINT 1, and MINT 31 methylation. IHC (Immunohistochemistry) assessed Ki67, CK20, hMLH-1, hMSH-2, and hMSH-6 protein expression. MSI analysis was performed on those polyps with adequate DNA remaining. Results. 124 HPs were identified 78 sporadic, 21 HNPCC, 25 familial, and the HNPCC group demonstrated no significant differences in overall methylation (P = .186 Chi²). The familial group demonstrated significantly less overall methylation levels (P = .004 Chi²). Conclusions. HPs that occur in HNPCC have no more worrying features at a molecular level than those patients with HPs in a sporadic setting.

1. Introduction

For many years hyperplastic polyps (HPs) have been considered innocuous lesions. Recent pathological and molecular observations have challenged this and given rise to the serrated adenoma carcinoma sequence. Prior to this, the adenoma carcinoma sequence was believed to be the mechanism by which most or all colorectal cancer (CRC) occurred; this sequence described a series of mutations in genes resulting in an increasingly dysplastic adenoma progressing to CRC over time. However, the reported accumulation of genetic changes described in the adenoma carcinoma sequence [1] does not wholly account for neoplastic transformation within the colon [2–4].

Mutation of the mismatch repair genes (most commonly MLH-1 and MSH-2) leading to MSI (microsatellite instability) in CRC is an alternative mechanism underlying tumour formation in patients with HNPCC [5]. It is now known that up to 15% of sporadic CRC also have MSI; not as a consequence of mutation in the mismatch repair genes but through a process of epigenetic changes to MLH-1 [6].

Epigenetics describes the silencing of key tumour suppressor genes through methylation of cytosine residues in the promoter regions of DNA [7]. Methylation occurs in up to 30% of CRC [8]. Hyperplastic polyps and serrated adenomas (SAs) have also been demonstrated to develop as a consequence of methylation. That methylation is present in HPs, SAs, and 30% of CRC without the series of mutations described in the adenoma carcinoma sequence has led to the suggestion of a serrated neoplasia pathway [7, 8].

A number of known genes are silenced in cancer by methylation including MLH-1, p16INK4a, MGMT, and MINT 1 and 31. Methylation affects gene expression in a graded fashion, a threshold of 15% promoter methylation is considered biologically significant [9–11]. The number of genes methylated can be described by CiMP (CpG island methylated phenotype): CiMP S, no methylation; CiMP L, one gene methylated, and CiMP H, more than one gene methylated [11, 12].

Most studies of sporadic HPs are either on patients with hyperplastic polyposis or selected HPs based on location in the colon or size. It is known that methylation is a frequent
event in sporadic HPs [13, 14]; however, the true incidence of methylation in HPs from a sporadic unselected cohort remains unknown. In the HNPCC screening program at Manchester Royal Infirmary 49% of polyps detected were HPs or SAs [15]. There are few papers describing the incidence of HPs in HNPCC and incomplete data regarding the molecular profiles of these lesions [16–18]. Mismatch repair mutations are considered the pathway by which cancer develops in patients with HNPCC. There is no evidence to date that those HPs that occur in HNPCC arise as a consequence of germline mismatch repair mutation and therefore should arise as a consequence of methylation like there sporadic counterparts. The methylation profile of HPs and SAs in patients with a family history of CRC is unknown. This study analyses methylation profiles of HPs which arise in patients with a greater than 1:10 empiric risk of CRC (familial group), patients with HNPCC, and patients who develop HPs sporadically. In addition Ki-67 and CK-20 immunostaining has been performed to analyse mucosal proliferation and differentiation as previously described [19–21].

2. Methods

2.1. Subjects and Recruitment. Patients were recruited with informed consent via the Manchester Royal Infirmary Endoscopy Unit (sporadic) and the regional genetics service HNPCC database (familial and HNPCC). Sporadic hyperplastic polyps were defined as polyps occurring in individuals without a family history of colorectal cancer; these contained an unselected 12-month cohort of specimens. Familial polyps were defined as polyps occurring in individuals with a greater than 1:10 empiric risk of CRC (familial group), patients with HNPCC, and patients who develop HPs sporadically. In addition Ki-67 and CK-20 immunostaining has been performed to analyse mucosal proliferation and differentiation as previously described [19–21].

2.2. Methylation Analysis. DNA extraction from paraffin embedded tissue was performed with a DNeasy kit (Qiagen Crawley, UK). Bisulfite modification of polyp DNA was performed using an EZ DNA methylation kit (Zymo Research, California, USA). Methylation analysis was performed with a combination of methylation specific PCR (MSP) for p16INK4a and MGMT, and MLH-1 and pyrosequencing for MINT 1 and MINT 31. Both methods have been previously well described [22–25]. Negative controls were generated from leukocyte DNA and positive controls from the SW48 cell line. The primers and PCR conditions for MSP are described in Tables 1–4. Greater than 15% methylation was considered biologically significant when quantifiable techniques were employed.

For methylation analysis of MINT 1 and MINT 31, primers were designed for the designated sequence as described by Toyota using pyrosequencing software provided by biotage (www.biotagebio.com), Table 5 and Figure 2 [11, 25]. The accuracy of pyrosequencing was confirmed by generating standard curves as previously described [25]. Pyrosequencing generated quantifiable results for methylation, a 15% threshold for methylation was considered biologically
Table 1: Primers used in the amplification of CpG islands from bisulfite-treated DNA by PCR (nested PCR) including their sequences and annealing temperature.

| Gene     | Genbank accession number | Sense primer                  | Antisense primer                  | Annealing temp (°C) |
|----------|--------------------------|-------------------------------|-----------------------------------|--------------------|
| P16INK4a | AF527803 NM_058195       | GGGTTTTTTTTAGAGG TATTGAGGAGTA | AAACAAACCCTC TACCCACCTAA         | 62                 |
| MGMT     | AL355531                 | GGGTTTTTTTTAGAGG TATTGAGGAGTA | CTCTCTACTTTTT CTCAAATCTCT         | 58                 |
| MLH-1    | U83845                   | TAGATTATTATTAGT AGAGGTATATAAG  | ATACCTTCAACC AATCACCCTCAATA       | 53                 |

Table 2: Primers used in the analysis of methylation at the amplified CpG islands of bisulfite treated DNA by PCR including their sequences and annealing temperature.

| Gene     | MSP reaction | Sense primer                  | Antisense primer                  | Annealing temp (°C) |
|----------|--------------|-------------------------------|-----------------------------------|--------------------|
| P16INK4a | Unmethylated | TTATAGAGGG TGGGTGGAGATTGT     | CAACCCCAACCC ACAACCATAA           | 64                 |
|          | Methylated   | TTATAGAGGGTGGG GGGAGATGC      | GACCCCCGAACC GGGACGGTAA           | 77.5               |
| MGMT     | Unmethylated | TTTGTGTTTTTGTAGT TTTAGGTTTTGTT | AACTCCACACTCT TCCAAAAACAAAAA     | 65                 |
|          | Methylated   | TTTGCAGGTTGCTTAG GTTTTCGC     | TTTGAGTTCGTTG AGGTTTTTCGC         | 70                 |
| MLH-1    | Unmethylated | TTAATAGGAAGAGT GGATAGTG       | TCTATAAATTACT AAATCTTCTCA         | 57                 |
|          | Methylated   | TTAATAGGAAGAGC GATAGC         | CTATAAAATTACTA AATCCTTTCG         | 60.5               |

Table 3: PCR conditions for the amplification step.

| Step                      | Temperature | Duration |
|---------------------------|-------------|----------|
| Initial denaturation      | 95°C        | 4 min    |
| Denaturation              | 95°C        | 1 min × 35|
| Annealing (Primer specific) °C | 1 min × 35  |          |
| Synthesis                 | 72°C        | 1 min × 35|
| Final extension           | 72°C        | 7 min    |
| Holding                   | 4°C         | —        |

Table 4: PCR conditions for the methylated/unmethylated step.

| Step                      | Temperature | Duration |
|---------------------------|-------------|----------|
| Initial denaturation      | 95°C        | 4 min    |
| Denaturation              | 95°C        | 30 sec × 35|
| Annealing (Primer specific) °C | 30 sec × 35  |          |
| Synthesis                 | 72°C        | 1 min × 35|
| Final extension           | 72°C        | 30 min   |
| Holding                   | 4°C         | —        |

2.3. Immunohistochemistry. Immunohistochemistry was performed on paraffin embedded tissues from 5 µM sections cut on a microtome and mounted on Surgipath positively charged slides (Peterborough, UK). Automated IHC was optimised using the TECHMATE 500 (DAKO, UK) and EnVision (DAKO, UK) detection system. Steam antigen retrieval was performed for 3 minutes in TRIS EDTA pH 9.0 for MLH-1 and MSH-6 and 0.01 mM EDTA for MSH-2, PMS-2, Ki 67, and CK 20. Antibodies were purchased from BD pharmenting, concentrations were as follows: MLH-1 1:75, MSH-2 1:200, MSH-6 1:20, Ki-67 1:200, and CK 20 1:200.

In total 167 hyperplastic polyps were analysed: 115 sporadic and 52 familial. A substantially larger number of sporadic hyperplastic polyp were analysed in comparison to methylation analysis since many of the smaller polyps either failed DNA extraction, were too small to consider for DNA extraction, or did not have adequate tissue in the block to perform DNA extraction. All polyps tested for methylation had IHC analysis with all 5 antibodies.

2.4. MSI. A panel of 5 markers: BAT25, BAT 26, NR-21, NR-24, and MONO-27 were used. These are all mono-nucleotide markers which are considered more sensitive for MSI than the original panel of markers recommended by the American
Joint Commission on Cancer [26, 27]. Microsatellite analysis was performed using previously described techniques [26, 27].

2.5. Statistics. Comparison between the sporadic and familial groups were made using the Fisher’s exact test where numbers were less than 5 or Chi² where numbers exceeded 5. SpSS and Excel software was used to perform statistical analysis. Correction for multiple comparisons were made.

3. Results

After tissue processing for methylation analysis there were 124 hyperplastic polyps with adequate amounts of DNA. There were 78 sporadic versus 25 familial polyps and 21 HNPCC polyps. Of the 124 polyps, 17 were from the right side of the colon (proximal to the splenic flexure) and 96 from the left side of the colon. For 11 polyps there were no records documenting position. The male-to-female ratio was 1 : 1, aged 22–78 years (median 47).

The overall incidence of methylation in all hyperplastic polyps tested were MLH-1 10%, p16 INK4a 26%, MGMT 19%, MINT 1 28%, and MINT 31 26%. When CiMP was calculated for the 124 hyperplastic polyps 36% (\(n = 45\)) were CiMP stable, 34% (\(n = 42\)) were CiMP low, and 30% (\(n = 37\)) were CiMP high. This results in an overall CiMP + rate of 64%.

For the HNPCC group there were no significant differences in the incidence of methylation in comparison to the sporadic group except for MINT 31 \(P = .046\) (Fisher’s exact test). These observations were further confirmed when comparing CiMP status where no significant differences were seen between CiMP low (\(P = .214\), CiMP high (\(P = .186\)), and CiMP + (\(P = .932\)) status (Figures 3 and 4).

The familial group demonstrated a significantly lower incidence of methylation of MINT 31 when compared to the sporadic cohort and nearly reached significance for MLH-1, p16\(^{INK4a}\), and MGMT. When CiMP status was plotted, significant differences were observed between CiMP stable, high, and overall CiMP +ve rates. CiMP low did not demonstrate a significant difference between either the HNPCC or familial group (Figures 3 and 4).

In total, 17 hyperplastic polyps were right sided. methylation was more frequent in the right side of the colon for p16\(^{INK4a}\), MINT 1, and MINT 31 (\(P = .019, .010\) and .012, resp., Chi²) but not for MGMT (\(P = .518\) Fisher’s exact test) or MLH-1 (\(P = .199\) Fisher’s exact test). Although over
double the amount of polyps from the right side of the colon had MLH-1 methylation detected by MSP (9% left versus 19% right) this difference did not reach significance ($P = .199$ Fisher’s exact test). However, when CiMP was compared between the left and right sides of the colon (Figure 5) CiMP high was more frequently encountered in HPs from the right side of the colon ($P = .040$ Chi$^2$).

### 3.1. MSI Hyperplastic Polyps

After methylation analysis there was enough DNA remaining to perform MSI analysis on 81 hyperplastic polyps. MSI-H was not detected in any of the hyperplastic polyps, MSI-L was detected in 3 (5.5%) of the sporadic and 1 (3.7%) of the HNPCC hyperplastic polyps, these differences were not significant (Fisher’s exact test 1.00). MLH-1 methylation was found in all four specimens demonstrating MSI-L.

#### 3.2. Details of Immunohistochemical Analysis of Hyperplastic Polyps

The results for IHC of the mismatch repair protein MLH-1, MSH-2, and MSH-6 can be seen in Table 6. None of the polyps demonstrated complete loss of staining. Weak staining was encountered with all three antibodies. This was believed to be related to poor tissue fixation in the majority of cases. However, those polyps which were methylated at the MLH-1 promoter region frequently showed weak staining with the MLH-1 antibody (10%; Figure 6). MSH-6 frequently showed weak staining in 47 (28%) of the hyperplastic polyps analysed (Figure 7).

The normal pattern of expression for Ki67 is in the base of the crypts and for CK20 the luminal surface of the crypts. Staining for Ki67 was predominantly basal or to the middle third of the crypts, more extensive staining to the outer third of the crypts was seen in 12/167 cases. Staining for CK20 was predominantly localised to the outer and middle third, although more extensive staining into the base of the crypts was seen in over one-third of cases Table 7.

### 4. Discussion

Analysis of methylation profiles of HPs from patients with a sporadic and familial risk of CRC has not previously been performed. This study has shown that in an HNPCC screening program, HPs that occur in those patients with confirmed mutations or HNPCC on clinical grounds have no more worrying features at a molecular level than those patients with HPs in a sporadic setting. Thus, those HPs that occur in patients with HNPCC could possibly be managed in
features at a molecular level than those patients with HPs in a sporadic setting. This study has also confirmed that methylation is a common biological event in sporadic HPs. There is a need for longitudinal studies and biological profiling of HPs to clarify the true risk of these lesions progressing to CRC.

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The safety of leaving HPs in HNPCC patients should be further confirmed as those in a screening program will receive alternate year colonoscopy. This new data may offer a novel contribution to the management of HNPCC patients. If so, patients with HNPCC who have HPs removed can be reassured these lesions are not manifestations of their condition. To validate these results a retrospective power study was performed looking for a 10% difference between the sporadic and HNPCC group for CIMP-H based on the provisional result from this study. This would require 376 patients in each arm to demonstrate a significant difference (that is, 0.05 with 80% confidence) if it exists. In addition should such a study be performed incorporating BRAF mutation analysis of polyp DNA along with methylation studies could give definitive conclusions with regards to the nature of HPs in HNPCC.

The true risk of developing CRC in association with HPs in either HNPCC or a sporadic setting is still unknown. Until a large prospective series, similar to the veteran a...
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