ORIGINAL ARTICLE

A study of genomic instability in early preneoplastic colonic lesions

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INTRODUCTION

The development of a colorectal polyp from a dysplastic adenoma has been well characterised. However, given these known pathways, it is difficult to explain the differential rates of progression of premalignant lesions and differences in behaviour of morphologically similar lesions. Heterogeneity for microsatellite instability (MSI) and promoter methylation in driving these phenomena forward may explain this; however, no previous analysis has examined this in detail at the gland level, the smallest unit of colorectal premalignant lesions. We aimed to carry out an analysis of gland level genomic instability for MSI and promoter methylation. MSI occurred significantly more frequently (20%) in colonic glands than has previously been observed in whole colorectal polyps. Significant promoter methylation was seen in MLH1, PMS2, MLH3 and MSH3 as well as significant heterogeneity for both MSI and promoter methylation. Methylation and MSI may have a significant role in driving forward colorectal carcinogenesis, although in the case of MSI, this association is less clear as it occurs significantly more frequently than previously thought, and may simply be a passenger in the adenoma-carcinoma sequence. Promoter methylation in MLH1, MLH3, MSH3 and PMS2 was also found to be significantly associated with MSI and should be investigated further. A total of 273 colorectal glands (126 hyperplastic, 147 adenomatous) were isolated via laser capture microdissection (targeted at regions of MLH1 loss) from 93 colonic polyps and tested for MSI, and promoter methylation of the DNA mismatch repair genes MLH1, MLH2, MLH3, MSH6, PMS2, MGMT and MLH3 via methylation specific multiplex ligation-dependent probe amplification. Logistic regression modelling was then used to identify significant associations between promoter methylation and gland histological type and MSI status.

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clinical criteria. Clearly, a comprehensive study is needed with clear criteria to understand the true distribution of heterogeneity within colonic lesions.

We hypothesised that genomic instability, specifically MMR deficiency, starts in very early colonic dysplasia, and is present in heterogeneous form in early tumours, resulting in MSI and this is associated with promoter methylation. We also hypothesised that the supposed hallmarks of genomic instability (that is, MSI associated with promoter methylation) occur in normal tissue and early tumours without gross evidence of underlying MMR deficiency.

RESULTS

A total of 91 sporadic polyps from 91 patients were obtained, and a total of 273 glands were microdissected (range 2–4 per polyp). The average age of patients was 60.9 years (range 33–91 years), with 48/91 (52.7%) being male and 43/91 (47.3%) being female. Of the 91 polyps, 45/91 (49.5%) were adenomatous (30/45 tubular and 15/45 tubulovillous) and 46/91 (50.5%) were hyperplastic. There were no serrated adenoma samples in the group. Of the 45 adenomatous polyps, 27/45 (60.0%) were mildly dysplastic, 5/45 (11.1%) were moderately dysplastic and 17/45 (37.8%) were severely dysplastic. In terms of the size of polyps in the hyperplastic poly group 2/46 (4.3%) were >1 cm in size, and in the adenomatous poly group 8/45 (17.7%) were >1 cm in size. After microdissection of the polyps into 273 glands, there were 126/273 (46.2%) glands from classical hyperplastic polyps available for study and 147/273 (53.8%) glands that were adenomatous, of which 81/147 (55.1%) were mildly dysplastic, 15/147 (10.2%) moderately dysplastic and 51/147 (34.7%) severely dysplastic.

Within the 91 polyps, 154/273 glands (57%) had heterogeneous loss of MLH1 expression (that is, the polyp had some MLH1 expression, but this particular gland did not), with the remaining 119/273 glands (43%) having no loss of expression. There was no homogenous loss of MLH1 expression (that is, the polyp had some MLH1 expression, but this particular gland did not), with the remaining 119/273 glands (43%) having no loss of expression. At the BAT25 locus, 155/273 (56.7%) glands were concordant for MLH1 loss and MSI, when looking at adenomatous and hyperplastic glands together. MSI was shown in Table 1, subdivided by dysplasia. In whole adenomatous polyps, 4/45 (8.9%) were MSI+ at the BAT25 locus and 3/45 (6.7%) were MSI+ at the BAT26 locus. For whole hyperplastic polyps, 4/46 (8.7%) were MSI+ at the BAT25 locus and 3/46 (6.5%) were MSI+ at the BAT 26 locus (see Table 1). On further analysis it was noted that 43/91 polyps were observed to be heterogeneous for gland level MSI within the sample polyp, that is, the glands studied within a single polyp were a mixture of MSI+ and microsatellite stable (MSS).

In order to examine the relationship between MLH1 expression status and MSI status, a 2 × 2 table of these variables was constructed (Table 2), for any one of BAT25 or BAT26. MSI status was significantly associated with the loss of MLH1 expression (Fishers exact, P = 0.001), although a small proportion of glands had MSI without loss of MLH1 expression. The association between MLH1 expression and MSI status is most pronounced when looking at adenomatous and hyperplastic glands together. 155/273 (56.7%) glands were concordant for MLH1 loss and MSI, however, glands were preselected for MLH1 protein expression, which could bias this figure. However, not all glands with MLH1 loss identified in the prescreening were MSI.

Twenty-one MSI+ glands were sequenced for BRAF codon 600 mutations. One out of eleven adenomatous glands (9%) and 1/10 (10%) hyperplastic glands had codon 600 mutations. The adenomatous gland had a c.1799 T > A, P.600 V > E mutation, and the hyperplastic gland a c.1798 G > C, P.6000 V > L mutation. These mutations were confirmed by repeat sequencing in both the forward and reverse directions. Interestingly the two mutations seen were not observed in other MSI+ glands within the same polyp.

Methylation specific multiplex probe ligand amplification (MS-MLPA) of colonic glands

In total, 114 glands (56 adenomatous glands and 48 hyperplastic glands), and 10 normal glands from normal tissue biopsies (in patients without polyps or cancer) were used in this study. Of the adenomatous glands, 20 were MSI+ and of the hyperplastic glands, 12 were MSI+. In total 114 MS-MLPA reactions were performed, with a failure rate of 28/114 (24.5%) all due to failure of hybridisation. In the 10 microdissected glands obtained from normal mucosal samples, promoter methylation was consistently observed in probes MLH1-a (10/10 samples), MSH2-b (10/10 samples), MGMT-c (8/10 samples) and MSH3-b (9/10 samples). There was sporadic methylation at three other probes in two other normal control samples—37% methylation in probe MSH6-a and MSH2-c in one sample and 67% methylation in probe MSH3-a in another sample.

Figure 1. Images of dysplastic adenoma (left) and dysplastic adenoma (right) demonstrating loss of normal MLH1 expression via immunohistochemistry (normal MLH1 expression in brown). Crypts harvested by laser capture microdissection are highlighted with a black line.
Because of these findings, it was felt that the analysis of 10 normal gland samples was adequate as the rate of background demethylation in normal samples was very low.

The relationship between MLH1 promoter hypermethylation and MLH1 protein expression via immunohistochemistry was also examined. (Table 3), and found that there was a significant association between MLH1 promoter methylation and loss of expression of MLH1. The MLH1 promoter region was said to be methylated if any of the MLH1 promoter region probes (excepting probe MLH1a that was methylated in all normal samples) had a methylation percentage exceeding our defined methylation threshold (20%). There was a significant association between MLH1 promoter hypermethylation and MLH1 protein expression loss (Fisher's exact, Bonferroni corrected $P = 0.009$). A table showing the relationship between methylation at each probe and gland type is shown in Supplementary Table 1.

In order to analyse the complex relationship between CpG island methylation, MSI status and histological type, a multivariate logistic regression model was constructed (see Supplementary Table S2 for full details). All MS-MLPA probes were entered into the regression model. Three separate models were constructed to ascertain, which methylated genes were significantly associated with MSI status; adenomatous polyps and hyperplastic polyps.

In examining, which MS-MLPA probes correlated with the presence of MSI within colonic glands, univariate analysis showed that methylation in MLH1 (probe B, $P = 0.026$) and demethylation in MSH3 (probe B, $P = 0.013$) and in MLH3 (probe B, $P = 0.026$) were all associated significantly with MSI status. In multivariate analysis only methylation of MLH1 (probe B, $P = 0.029$) remained significant. For adenomatous glands, univariate analysis showed a significant association with methylation in PMS2 (probe B, $P = 0.013$), however, after multivariate analysis demethylation of MLH1 (probe E, $P = 0.048$) and methylation of MLH3 (probe A, $P = 0.048$) became significant (Table 4).

For hyperplastic glands, on univariate analysis methylation in PMS2 (probe C, $P = 0.034$) and MLH3 (probe B, $P = 0.04$) was significant. On multivariate analysis demethylation in MSH3 (probe A, $P = 0.026$) and MLH3 (probe B, $P = 0.003$) and methylation in MLH3 (probe B, $P = 0.024$) and PMS2 (probe B, $P = 0.029$) were found to be significantly associated with hyperplastic glands (Table 4).

In order to explain the MSI positivity in glands that were not methylated at MLH1, the regression model was repeated, this time omitting the probes that were observed to be relevant previously (MLH1 probe B) or known to be methylated in normal tissue (MLH1 probe a, MLH1 probe f, MSH2 probe b and MLH3 probe a). In a univariate model (Supplementary Table S3), several probes were significant including methylation of PMS2 (probe A, $P = 0.024$), de-methylation of MGMT (probe A, $P = 0.037$) and MLH3 (probe B, $P = 0.022$).

**DISCUSSION**

This study has examined the presence and rates of MSI in isolated colorectal glands, showing that MSI does exist in isolated glands,
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These findings suggest that there may be other loci that influence MSI status and it is possible that these may be influenced by methylation in other MMR genes. Statistical analysis of this demonstrates a complex relationship, with promoter methylation of PM22, MGMT and MLH3 being potentially significant in initiating MSI. The heterogeneity for methylation observed within glands in our study is widespread, leading to the possibility that the methylation seen is a random phenomenon that may or may not trigger MSI leading to accelerated polyp development.

A weakness of this study is that replicates were not obtained for every sample analysed via MS-MLPA, and that the MS-MLPA results were not verified by another technique such as bisulfite sequencing or pyrosequencing. This was not done because of the limitations of the small amounts of DNA inherent to laser capture microdissection. MS-MLPA has also been shown to be robust and reproducible in prior studies, and thus was considered sufficiently reliable to be used in this manner. In addition, the use of both hyperplastic and adenomatous polyps in this study could potentially be criticised as biasing the results, as two separate pathways are thought to be involved in the initiation of these polyps. However, we feel that the relationship between MSI promoter methylation and MSI is likely to be ‘polyp independent’ as this is the only biologically plausible way in which this could occur.

In conclusion, MSI is present in individual colonic glands, and at a higher rate than previously demonstrated for whole polyps. The mechanism for this is not completely clear, but may partly be caused by MLH1 promoter methylation leading to inactivation of the gene as demonstrated in colorectal cancer cells in previous studies. This may be of clinical importance, as there is an increasing use of molecular stratification of tumours into MSI/MSS, and this study suggests that this phenomenon may be heterogeneous. If this is the case, this will need to be carefully accounted for in any study of the link between MSI and clinical outcome.

This study also raises issues regarding the clonality of colorectal polyps. Our results would suggest that polyclonality exists in colorectal polyps, at least for MSI and for methylation. Two possible scenarios exist with regards to this finding, first that multiple pathways, in which a polyp arises can exist within the same polyp and contribute to the progression of the polyp, and that a single pathway becomes dominant towards the end of progression of the polyp. Alternatively, it is possible that this observed heterogeneity is of no consequence and that a single pathway is still dominant and the observed heterogeneity are merely ‘passenger’ events. Clearly further investigation is needed of this intriguing phenomenon.

There is a clear link between MMR gene promoter methylation and MSI status; however, it is unclear, excluding MLH1 methylation, which promoters cause a tendency to MSI+. Other MMR genes may have a role as suggested by this study. Little is known about the functional relevance of these genes and further study is needed to ascertain their relevance to colorectal cancer.

MATERIALS AND METHODS

Patients were recruited as from the SW London Colonoscopic Surveillance study (MREC 07/06860/09). Patients who were >18 years old, of Caucasian background, had at least one polyp at colonoscopy and with no family history or personal history of colorectal cancer or endometrial cancer (to the second degree level) were chosen.

Formalin-fixed, paraffin-embedded blocks were obtained and serial sections cut for H&E staining and immunohistochemistry and onto PEN laser capture slides for laser capture microdissection. Laser capture slides were stained with a 0.5% solution of methyl green dye (Sigma-Aldrich, Dorset, UK) to highlight cellular structure for microdissection. Immunohistochemistry against MLH1 was carried out using an indirect secondary method using mouse antihuman MLH1 monoclonal antibody at 1:50 dilution for 1 h at room temperature (BD Pharmingen G168-15, no.550838, Oxford, UK). MLH1 expression was scored as present or lost in either individual glands or the whole polyp when compared with the control

and at a rate (20%) higher than has been previously reported in whole polyps tested for MSI.

Previous studies have shown the rates of MSI of ~2–15%. However, previous attempts to analyse MSI in polyps may have reported lower rates of MSI because only a few glands within a polyp may be MSI+ and their signal is overwhelmed by the majority MSS DNA present within the polyp sample.

Of note is the level of heterogeneity for MSI seen in colorectal glands. In this study, just under 50% of all polyps showed heterogeneity for MSI (Table 1b). This suggests that MSI may exist at a higher rate than previously observed. Accelerated progression to cancer has been observed in tumours where MSI exists compared with MSS tumours, although this has not been observed consistently, and one possible explanation for this phenomenon is the presence of a clone of microsatellite unstable glands within an apparently MSS polyp. This may confer a selective growth advantage towards neoplasia (because the MSI crypt has a survival advantage of MSS crypts, leading to it becoming the dominant clone) even if the eventual tumour does not appear to be microsatellite unstable. It is also possible that MSI is a background phenomenon and is only present in but does not participate in the progression to carcinogenesis. Hypermethylation of the MLH1 promoter region has been seen in normal colonic mucosa at a very low level, but has not been associated with MSI.

A possible further study would be to carry out MS-MLPA analysis of the whole colonic polyps to ascertain whether methylation status at the crypt level differs to that of the whole polyp.

The concordance between MLH1 expression and methylation is only ~60% for all glands in our study, lower than would be expected if MLH1 methylation was solely responsible for MSI. This differs from the findings of Kloor et al. and Yurgelun et al., however, their studies examined normal mucosa and polyps from Lynch syndrome carriers only. The mechanism by which this occurs in sporadic lesions may be different and may be related to methylation of other MMR gene CpG islands within these glands. We also noted heterogeneity for BRAF mutation status within the same polyp. This could represent the heterogeneity already seen for MSI within the polyp, or these BRAF mutations could simply be random passenger mutations occurring during polyp progression.

The hyperplastic glands in this study demonstrate an extensive pattern of methylation in the DNA repair genes, specifically with methylation occurring within the CpG islands of PM22, MLH3 and most strongly, MSH3. Loss of MLH3 expression has been shown to lead to MSI and MSH3 is known to form a heterodimer with MSH2 and participate in DNA repair.

![Table 4. Multivariate stepwise regression model for gland level MSI status, adenomatous gland and hyperplastic gland vs MLPA probes](image-url)

| Probe     | OR   | 95% CI     | P-value (Bonferroni corrected) |
|-----------|------|------------|-------------------------------|
| MSI status |      |            |                               |
| MLH1-b    | 3.46 | 1.14–10.56 | 0.029                         |
| Adenomatous gland | | | |
| MLH1-e    | 0.20 | 0.04–0.99  | 0.048                         |
| MLH3-a    | 5.01 | 1.01–24.8  | 0.048                         |
| Hyperplastic gland | | | |
| MSH3-a    | 0.19 | 0.04–0.82  | 0.026                         |
| MSH3-b    | 0.20 | 0.07–0.57  | 0.003                         |
| MLH3-b    | 4.96 | 1.23–20.03 | 0.024                         |
| PMS2-b    | 5.20 | 1.19–22.83 | 0.029                         |

Abbreviations: CI, confidence interval; MSI, microsatellite instability; MLPA, multiplex ligation-dependent probe amplification; OR, odds ratio.
DNA was extracted from laser capture microdissected glands using a PicoPure DNA (Molecular Devices Inc., Sunnyvale, CA, USA), kit was added and DNA was extracted according to the manufacturer’s instructions. An ethanol co-precipitation method was used to purify DNA using a glycogen carrier.

Analysis of MS-MLPA was carried out using BAT25 and BAT26 primers (sequences available on request) in a standard PCR reaction. MS-MLPA analysis were inspected visually to ensure that they passed quality control criteria. PCR products were analysed on an ABI 3730xl automated capillary sequencer. Pattern of fragment analysis was assessed visually to ensure that they passed quality control according to the manufacturer’s instructions. If any peaks were present in the blank water well, the sample plate was said to have failed. A custom-made Microsoft Excel spreadsheet (available on request) was used to quantify methylation at each allele by carrying out intrasample normalisation of each informative probe to several reference probes. Methylation was expressed as a percentage at each allele.

All statistical analyses were performed with STATA 11.1 (StataCorp, TX, USA). A observed frequency of methylation in normal tissue of 5% was assumed, based on the literature with a change in methylation rate in polyp samples to 50%, therefore it was calculated a minimum sample size of 23 would be needed in the adenoma and hyperplastic polyp groups each, assuming 90% power and a significance level of 0.05. In order to analyse rates vs levels of DNA methylation in individual colonic crypts reveal aging and cancer-related field defects in the morphologically normal mucosa. Carcinogenesis 2010; 31: 1158–1163.

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