K-RAS, B-RAF and GNAS Gene Status and Immunohistochemistry Analysis of Mucinous Neoplasm of Appendix

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Research
Abstract

AIM

Low grade appendiceal mucinous neoplasm (LAMN) and serrated lesions are sometimes hard to differentiate from morphology. We try to characterize them from the immunohistochemical and molecular perspective and to reflect the difference between them.

METHODS

25 appendix neoplasm including 13 LAMNs, 12 serrated lesions were selected from January 2013 to December 2014. Immunohistochemical analyses for cytokeratin 20, MUC6, MUC5AC, MUC1, Ki-67, P53 and mismatch repair (MMR) proteins including MLH1, PMS-2, MSH-6, MSH-2 were performed. Microsatellite instability (MSI) status was also evaluated. Besides, we detect K-RAS, B-RAF and GNAS gene mutation status of these lesions.

RESULTS

Immunohistochemically, 83.3% serrated lesions showed scattered CK20 staining in the deep crypt, which was less so for LAMNs. As for mucin expression, MUC5AC had slightly higher positive rate in LAMNs than in serrated lesions. MUC1 was significantly higher expressed in LAMNs than in serrated lesions. 46.1% LAMNs have P53 expression in deep crypt, while P53 was negative in the deep crypt of serrated lesions. 58.3% serrated lesions had deficient MMR protein expression pattern compared to 23.1% of LAMNs. B-RAF mutation was detected in 3 cases, all were serrated lesions. K-ras and GNAS mutation was detected in both LAMNs and serrated lesions.

CONCLUSION

Immunohistochemical panel comprising markers such as CK20, MUC5AC, MUC1, Ki-67 and P53, with genotyping covering hotspots of the KRAS, BRAF and GNAS genes can help the differential diagnosis of low grade appendix neoplasm.

Introduction

Low grade appendiceal mucinous tumor (LAMN) and appendiceal serrated lesion are low grade appendix neoplasms that share similar clinical presentations whereas variable biological behaviors, from a benign indolent course to a relatively aggressive malignant process.

LAMN is an appendiceal neoplasm characterized by replacement of the normal appendiceal mucosa by a filiform villous mucinous epithelial proliferation. Pseudomyxoma peritonei (PMP) that originated from
ruptured mucinous appendiceal tumor will have a low 5-year survival rate[1]. Therefore, it is necessary to differentiate LAMN from the other benign lesions, such as serrated lesion, before it ruptured.

From the authors’ experience, LAMN, appendiceal serrated lesion are difficult to differentiate, due to overlaps in the morphology. Several research groups have used immunohistochemical analyses (cytokeratin20, Ki-67, MUC6, and β-catenin) to differentiate different types of serrated polyps [2–4]. Yet, whether the panel of CK20, Ki-67, and mucins are useful in differentiating serrated and non-serrated lesions remains poor clarified. It is also established that serrated neoplasm from colon and rectum are precursor lesions for sporadic MSI-H cancers and most sessile serrated adenoma/polyps (SSA/Ps) harbor mutations in BRAF (up to 90%) [5, 6]. Since serrated polyps in the appendix showed similar morphology to their colorectal counterparts, there are many reports focusing on the genetic alterations in appendix such as K-ras, BRAF and GNAS, but few focus on dMMR status and relevant molecular changes.

In this study, we compared the immunophenotypic and molecular profiles of different low-grade neoplasms of appendix to better understand the difference between the two lesions.

**Materials And Methods**

**Patients and Diagnosis**

We searched the pathology electronic documentations of the Peking Union Medical College Hospital (2013 to 2014) for appendiceal lesions using the following keywords: low grade appendiceal mucinous neoplasm, hyperplastic polyp, sessile serrated adenoma, serrated adenoma, and tubular adenoma. Corresponding H&E slides were retrieved and reviewed by 4 pathologists independently by consensus to the following categories: serrated lesions and LAMN. Serrated lesions included HPs and Serrated desplasia, which were diagnosed based on distorted crypts with serration crypt dilatation extending to crypt bases with or without cytologic dysplasia. LAMNs were diagnosed by a filiform villous mucinons type or columnar epithelial cells with nuclear pseudostratication growing on a fibrotic submucosal tissue.

**Immunohistochemical analysis**

Immunohistochemical analyses were performed on 4-µm sections made from a representative tissue block from each of the cases on Autostainers. The antibody information including clone, vendor, dilution, positive pattern and the proper antigen retrieval condition were listed in Supplementary Table 1. Positive and negative controls were included in each batch of staining. For MMR components (MLH1, PMS-2, MSH-6, MSH-2), tumour stromal cells and inflammatory cells were used as internal controls, loss of MMR protein expression was determined as defective MMR (dMMR).

**MSI status analysis**
Microsatellite analysis was performed as described previously using Microsatellite Instability Detection Kit (Microread, China) [7]. In brief, DNA was first extracted from paraffin-embedded tissue blocks and then conduct multiplex fluorescent polymerase chain reaction (PCR). MSI locus recommended by NCCN guideline such as NR-21, NR-24, NR-27, BAT-25, BAT-26, Mono-27 was amplified and detected. New PCR peaks appeared in two or more of six locus was regarded as high-frequency MSI (MSI-H), in one of six locus as low-frequency (MSI-L), and no new peaks in six locus as microsatellite stable (MSS).

BRAF, KRAS and GNAS mutation analysis

Appendiceal lesions were enriched by macro dissection from 4-micron sections. DNA was extracted using the DNA extraction kit (AmoyDx® FFPE DNA Kit, Cat No. ADx-FF01, Amoy Diagnostics Co, China). KRAS and BRAF mutation analyses were accomplished using commercially available ARMS-PCR based assays (AmoyDx® BRAF 600 and KRAS mutations detection kit ADx-BR04-R, ADx-KR05, Amoy Diagnostics Co, China) for 25 cases according to the manufacturer’s instructions. The coverage of the kits was detailed in Supplementary Table 2. GNAS mutation was analyzed by Sanger sequencing. The primers were designed as follows to amplify DNA fragment including exon 201 of the gene. Forward: AGACCTTTGCTTTAGATTGGC; reverse: CTTACTGGAAGTTGACTTTGTCC. This primer pair yielded a PCR product of 157 bp. The purified fragments were subjected to direct sequencing on the Genetic Sequencer ABI3500 (Applied Biosystems, Grand Island, USA) using the BigDye® Direct Sanger Sequencing Kit following the manufacturer’s protocol.

Statistical analysis

Statistical analysis was performed by SPSS 17.0 (SPSS, Chicago, IL, USA). Differences in immunohistochemical indexes and gene mutations between groups were assessed with $\chi^2$ test. All statistics were assessed using two-sided tests with a $P < 0.05$ being considered statistically significant.

Results

LAMN presented female predilection [8], the incidence ratio of female to male is 11/2. The average ages for the two groups were 63.5 and 66.7 years, respectively. One case of LAMN was coincidentally found in colon carcinoma resection. 6 out of 13 LAMNs had coexisting PMP. For serrated lesions, only one was found by appendectomy due to acute appendicitis. The patient characteristics were summarized in Table 1.
Table 1
Clinicopathologic features of patients with appendiceal lesions

| Clinicopathologic features | LAMN | Serrated lesions |
|---------------------------|------|-----------------|
| Total no. of cases        | 13   | 12              |
| Gender (F/M)              | 11/2 | 7/5             |
| Average age               | 63.5 | 66.7            |
| Lesion size (mm) (range)  | 2 × 1 × 1 cm-4.2 × 1.5 × 1.5 cm | 0.3 cm-4.7 × 0.6 × 0.6 cm |
| Gross cystic dilatation   | 8    | 0               |
| Gross luminal mucin       | 13   | 12              |
| Gross rupture             | 4    | 0               |
| Polyp growth pattern      | 1    | 2               |
| Discrete polyp            | 1    | 2               |
| Associated lesions        |      |                 |
| Colon carcinoma           | 3    | 4               |
| IBD (UC or CD)            | 0    | 0               |
| Ovary carcinoma           | 0    | 3               |
| PMP                       | 9    | 0               |
| Appendicitis              | 1    | 1               |

IBD: inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn's disease; PMP: pseudomyxoma peritonei

Immunohistochemically, CK20 was expressed in the surface epithelium in two types of lesions. But in the deep crypts, CK20 was positive in 10 of 12 serrated lesions and 3 out of 12 LAMNs (Fig. 1). The difference was statistically significant (Table 2). Five and six LAMNs and serrated lesions showed focal or scatter positivity for MUC-6, respectively. All LAMN cases and 11 of 12 cases of serrated lesions were MUC5AC positive. Regarding to MUC1, 12 out of 13 LAMNs were positive, at the mean time 4 serrated lesions were immunoreactive. None of the serrated lesions were positive for P53, while 6 LAMNs were P53 positive. In the two entities, Ki-67 were mostly located in the deep crypts, while in four LAMNs and one serrated lesions, the staining went to the upper part of the crypt.
Table 2
Immunohistochemical features of study patients with appendiceal lesions

| Immunohistochemical features | LAMN                          | Serrated lesions                  | P Value |
|-----------------------------|-------------------------------|-----------------------------------|---------|
| Total no. of cases          | 13                            | 12                                |         |
| CK20                        | Diffuse positive, 3 with crypt scatter positive | Diffuse positive, 10 with crypt scatter positive | 0.005* |
| MUC6                        | 5 focal positive              | 6 focal or scatter positive       | 0.695   |
| MUC5AC                      | 13 diffuse positive           | 11 diffuse positive               | 0.458   |
| MUC1                        | 12 focal surface epithelium positive | 4 focal surface epithelium positive | 0.008* |
| P53                         | 6 positive                    | 0 positive                        | 0.015*  |
| Ki-67                       | 4 case positive both in the crypt and in surface epithelium, 9 with crypt positive | 1 case irregular positive, 11 cases with crypt positive | 0.322   |

P Value was calculated using χ² test.

* statistically significant different

In terms of the four mismatch repair proteins, MLH1, PMS2, MSH2 and MSH6, LAMN, serrated lesions both had dMMR. The difference was statistically significant (P = 0.047). Immunohistochemical interpretations of all markers were summarized in Table 3. Three of thirteen LAMN have loss of MSH6 in the villous structure, in serrated lesions, 7 of 12 serrated lesions have dMMR in the protein level including loss of MSH6, PMS2 and MLH1, although direct analyses of representative MSI loci showed none of serrated lesions of the appendix were MSI-H (Table 3). Results of mutational analyses were listed in Table 4. Four types of K-ras mutation were detected, including G12D, G12V, G13D and G12C. KRAS mutation were detected in 12 LAMNs and and 5 serrated lesions. Among them, G12D was the most common genotype, distributing in eight LAMNs and two serrated lesions. Of note, the only one G12C mutation was occurred in a serrated lesion. The difference in the frequencies of KRAS mutation in the two groups was statistically significant (P = 0.011). Only serrated lesion carried BRAF mutation in the present case series (3/12). GNAS mutation was found in 5 LAMN cases, 2 serrated lesions, respectively. The difference in GNAS mutation profile among groups was not significant (P = 0.378). Interestingly, all of GNAS mutation (8 of 8 cases) in both lesions was found to coexist with KRAS mutation. (Fig. 2G).
|                  | LAMN | Serrated lesions | P Value |
|------------------|------|-----------------|---------|
| Total case       | 13   | 12              |         |
| dMMR             | 3    | 7               | 0.047*  |
| PMS2             |      |                 |         |
| All preserved    | 8    | 4               |         |
| MHL1             |      |                 |         |
| All preserved    | 3    | 3               |         |
| MSH6             |      |                 |         |
| 3 deficient      | 8    | 8               |         |
| MSH2             |      |                 |         |
| All preserved    | 3    | 3               |         |
| MSI-DNA          |      |                 | 1.000*  |
| MSS              | 12   | 11              |         |
| MSI-H            | 0    | 0               |         |
| MSI-L            | 1    | 1               |         |

P Value was calculated using χ² test.

* statistically significant different
### Table 4
Molecular features of study patients with appendiceal lesions

| molecular features | LAMN | Serrated lesions | P Value |
|-------------------|------|-----------------|---------|
| Total case        | 13   | 12              |         |
| K-RAS(Cases /Mutation type) | 12   | 5               | 0.011*  |
| G12V/35G > T      | 2(1 case co-exist GNAS mutation) | 0       |
| G12D/35G > A      | 8(3cases co-exist GNAS mutation) | 2(1 case co-exist GNAS mutation) |
| G13D/38G > A      | 2(1 case co-exist GNAS mutation) | 2(1 case co-exist GNAS mutation) |
| G12C/34G > T      | 0    | 1               |         |
| No mutaion        | 1    | 7               |         |
| B-RAF             | 0    | 3(with no other mutation) | 0.096   |
| G-NAS(Cases /Mutation type) | 5    | 2               | 0.378   |
| 602G>A            | 2    | 2               |         |
| 601C>T            | 3    | 0               |         |
| No mutation       | 8    | 10              |         |

P Value was calculated using χ² test.

* statistically significant different

### Discussion

LAMN, and serrated lesion are two major appendiceal premalignant lesions, they morphologically resemble each other. Sometimes, when the lesion is small and is not as typical, it is difficult to distinguish them. We try to look for their differences from the immunohistochemical and genetic point of view.

Torlakovic and his colleagues used CK20 and Ki-67 immunohistochemistry to appreciated the aberrant maturation and proliferation of the crypts in serrated lesions[2]. In a large proportion of serrated lesions in our study, scattered CK20 positivity in the deep crypt was observed, which wasn't different from Torlakovic's findings. This phenomenon was also observed by Andrew M. Bellizzi et al[9]. Of note, a significantly smaller fraction of LAMN also showed this staining pattern, which has not been previously reported. So, CK20 positivity in the deep crypt was charatracstic of serrated lesions in appendix.
In the present study, 6 in 13 of LAMN cases showed P53 scattered positive in deep crypt. This rate was relatively higher than results of Hara et al [10]. Besides, all the P53 positive LAMNs patients were female and had concomitant PMP, which was in concordance with existing literatures, P53 over-expression in PMP of appendiceal origin was significantly related to female sex, spreading to the abdominal cavity, and worse survival for patient [11]. In serrated lesions, P53 were all negative, even in cases with epithelial dysplasia, this result was the same as Yantiss et al [12], only 1 serrated adenoma showed more than 10% of the surface epithelium positive. This may imply that serrated lesions in appendix was mostly low grade, the reason might be they lack of mucus secretion and lower p53 expression.

Ki-67 was mostly expressed in deep crypt of LAMN (9/13) and serrated lesions (11/12), while four LAMNs and one serrated lesions have Ki-67 staining in surface epithelium as in conventional colon adenomas.

Different types of mucins expression with varying strength has been reported in serrated lesions and adenomas of the colon and rectum [13–15]. We therefore chose three types of mucins, including MUC6, MUC5AC, and MUC1. MUC6 was already showed to associate with morphologic serrated features of appendix in the study of Bellizzi et al [9]. Our result showed that LAMN and serrated lesions both can have MUC6 expression (5/13, 6/12), although only in focal area.

LAMN shared similarity with pancreatobiliary subtype of pancreatic intraductal papillary mucinous neoplasm (IPMN), which is usually positive for MUC5AC and MUC1 and negatively for MUC6 [16, 17]. MUC5AC and MUC1 were indeed highly expressed in LAMN in our cases. Besides, serrated lesions also have high MUC5AC expression and 6 of 12 serrated lesions have focal MUC6 expression as LAMN. This indicated that both LAMN and serrated lesions of appendix can have MUC5AC and focal MUC6 expression, and a combination of MUC5AC and MUC1 might help us to distinguish LAMN from other lesions of the appendix. The possible mechanism may be GNAS mutation, which was characteristic of LAMN [18], could induce MUC5AC and MUC2 expression in colorectal cancer cell lines, so in LAMN, MUC5AC is all positive in both crypt and surface epithelium. Focal MUC6 expression in both LAMN and serrated lesions may explain their morphologic similarities. Besides, in Mesa’s research [19], MMR deficiency in appendix neoplasms showed a correlation with MUC5AC and MUC6 expression. In our LAMN and serrated cases, 11/24 that have MUC5AC expression also have dMMR, and 6/11 that have focal MUC6 expression also have dMMR.

Serrated lesions of colon carcinoma tend to have higher rate of microsatellite instability [20]. In concordance with colon carcinoma, our result also showed that serrated lesions in appendix are prone to have dMMR than LAMN. 7 of 12 serrated lesions have dMMR in the protein level including loss of MSH6, PMS2 and MLH1, although Direct analyses of representative MSI loci showed none of serrated lesions of the appendix were MSI-H. Yantiss et all [12] also reported that incomplete loss of MLH1 protein in serrated polyps of appendix was not accompanied by MSI-H. In our cases, three LAMNs have loss of MSH6 in the villous structure but no loss of MSH2, although this type was rarely reported in LAMN, only in MSI-high
appendiceal carcinoma[21]. This indicated that LAMN can also have microsatellite instability, even though they are at an early stage.

KRAS mutation in LAMN was significantly higher than in serrated lesions, with 12 of 13 cases having different type of mutations. This is consisted with the previous reports[18, 12, 22, 23]. In their study, 94% cases of LAMN were KRAS mutated and the most frequent mutation type was 35G > A. 5 of 12 serrated lesions also have K-ras mutations, which indicated that LAMN and serrated lesions may share similar oncogenic pathways.

Several articles reported a considerable proportion of LAMNs harboring GNAS mutation[24, 18]. In our study, only 5 of 13 (38.5%) LAMN cases exists GNAS mutation. This rate was higher than the serrated lesions. This relatively low rate of GNAS mutation in LAMNs might be due to the low sensitivity of Sanger sequencing and limited exon coverage. Alakus and other researchers [24–27] also proposed that co-existing mutations of KRAS and GNAS were characteristic to LAMN and KRAS mutation occurs earlier in the course of tumorigenesis. This pathway was also shared by IPMN. In the present study, 8 cases, including both LAMN and serrated lesions, that have GNAS mutation, also have KRAS mutation co-exist. These may have indicated that GNAS mutation was prone to happen following the KRAS mutation.

BRAF mutation was only presented in 3/12 cases of serrated lesion. Rish K. Pai et al [28] found serrated lesions in appendix harbor more KRAS mutations instead of BRAF mutations, by which they regarded serrated lesions in appendix as a distinct entity from their counterparts in the colon, which was supposed to have more BRAF mutation. Other researchers[25] reported higher rate of BRAF mutation of the serrated lesion. Our result also indicated a slightly more KRAS mutation (5/12) in serrated lesions than BRAF mutation (3/12) in the appendix and our histological criteria was almost the same with them. BRAF mutation was only confined to serrated lesions in the present study, suggesting that BRAF could be used as a specific yet insensitive marker for serrated lesion in appendix. Similar as the colorectal counterparts, serrated lesion in appendix can have dMMR and BRAF mutation.

In conclusion, LAMN and serrated lesion each had their characteristic immunohistochemical expression and molecular mutations, LAMN was both MUC1 and MUC5AC positive and harbors both KRAS and GNAS mutation, and they can also have loss of MSH6 immunoexpression and also have high Ki67 index in the surface epithelium. Serrated lesions can also have dMMR, with MUC5AC and MUC6 focal expression and they were all p53 negative and they also harbor KRAS mutation. Combination of these different markers could assist the differentiation.

**Abbreviations**

LAMN: Low grade appendiceal mucinous neoplasm; MMR: mismatch repair; PMP: Pseudomyxoma peritonei; SSA/Ps: sessile serrated adenoma/polyps; dMMR: defective MMR; PCR : polymerasechain reaction; MSI-H : high-frequency MSI; MSI-L : low-frequency MSI; MSS : microsatellite stable
Declarations

Author Contributions Statement

Xinyu Ren, Weixun Zhou and Zhiyong Liang reviewed the case together. Xinyu Ren and Yin Cheng wrote the article. Tao Lu and Yan Wu did the mutation test. Junyi Pang did the Immunohistochemical test.

- Conflict of interest
- The submission was approved by all authors and none of the authors has any potential financial conflict of interest related to the manuscript.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study obtained the approval of the ethics committee of Peking Union Medical College Hospital. Written informed consent was obtained from each patient.

Consent for publication

Not applicable. All authors read and approved the final manuscript.

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**Tables**

**Table 1** Clinicopathologic features of patients with appendiceal lesions

| Clinicopathologic features | LAMN        | Serrated lesions |
|----------------------------|-------------|------------------|
| Total no. of cases         | 13          | 12               |
| Gender(F/M)                | 11/2        | 7/5              |
| Average age                | 63.5        | 66.7             |
| Lesion size (mm)(range)    | 2x1x1cm-4.2x1.5x1.5cm | 0.3cm-4.7x0.6x0.6cm |
| Gross cystic dilatation    | 8           | 0                |
| Gross luminal mucin        | 13          | 12               |
| Gross rupture              | 4           | 0                |
| Polyp growth pattern       | 1           | 2                |
| Discrete polyp             | 1           | 2                |
| Associated lesions         |             |                  |
| Colon carcinoma            | 3           | 4                |
| IBD (UC or CD)             | 0           | 0                |
| Ovary carcinoma            | 0           | 3                |
| PMP                        | 9           | 0                |
| Appendicitis               | 1           | 1                |

IBD: inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn’s disease; PMP:pseudomyxoma peritonei
Table 2  Immunohistochemical features of study patients with appendiceal lesions

| Immunohistochemical features | LAMN                          | Serrated lesions                          | P Value |
|------------------------------|-------------------------------|-------------------------------------------|---------|
| Total no. of cases           | 13                            | 12                                        |         |
| CK20                         | Diffuse positive, 3 with crypt scatter positive | Diffuse positive, 10 with crypt scatter positive | 0.005*  |
| MUC6                         | 5 focal positive              | 6 focal or scatter positive               | 0.695   |
| MUC5AC                       | 13 diffuse positive           | 11 diffuse positive                       | 0.458   |
| MUC1                         | 12 focal surface epithelium positive | 4 focal surface epithelium positive      | 0.008*  |
| P53                          | 6 positive                    | 0 positive                               | 0.015*  |
| Ki-67                        | 4 case positive both in the crypt and in surface epithelium, 9 with crypt positive | 1 case irregular positive, 11 cases with crypt positive | 0.322 |

P Value was calculated using $\chi^2$ test.

* statistically significant different

Table 3  Immunohistochemical and molecular Micro-satellite state
|                | LAMN     | Serrated lesions | P Value |
|----------------|----------|------------------|---------|
| Total case     | 13       | 12               |         |
| dMMR           | 3        | 7                | 0.047*  |
| PMS2           | All preserved | 8 preserved, 4 deficient |         |
| MHL1           | All preserved | 3 deficient       |         |
| MSH6           | 3 deficient | 8 deficient       |         |
| MSH2           | All preserved | All preserved    |         |
| MSI-DNA        |          |                  | 1.000*  |
| MSS            | 12       | 11               |         |
| MSI-H          | 0        | 0                |         |
| MSI-L          | 1        | 1                |         |

P Value was calculated using $\chi^2$ test.

* statistically significant different

**Table 4** Molecular features of study patients with appendiceal lesions
| molecular features | LAMN | Serrated lesions | P Value |
|-------------------|------|-----------------|---------|
| Total case        | 13   | 12              |         |
| K-RAS(Cases /Mutation type) | 12   | 5               | 0.011*  |
| G12V/35G>T        | 2(1 case co-exist GNAS mutation) | 0       |         |
| G12D/35G>A        | 8(3cases co-exist GNAS mutation) | 2(1 case co-exist GNAS mutation) |         |
| G13D/38G>A        | 2(1 case co-exist GNAS mutation) | 2(1 case co-exist GNAS mutation) |         |
| G12C/34G>T        | 0    | 1               |         |
| No mutation       | 1    | 7               |         |
| B-RAF             | 0    | 3(with no other mutation) | 0.096   |
| G-NAS(Cases /Mutation type) | 5    | 2               | 0.378   |
| 602G>A            | 2    | 2               |         |
| 601C>T            | 3    | 0               |         |
| No mutation       | 8    | 10              |         |

P Value was calculated using χ² test.

* statistically significant different
LAMN and serrated lesions of appendix. Both two lesions showed diffuse CK20 expression. Scattered CK20 positive in deep crypt could be seen in serrated lesion. MUC1 and MUC6 could be detected locally in LAMN and serrated lesions. MUC5AC were diffuse positive in LAMN and serrated lesion, both in the crypt and in surface epithelium. P53 and Ki67 were positive only in base of crypt.

**Figure 2**

A H&E stain of a case of serrated lesion (40x). B MLH1 staining positive in tumour. C Positive MSH2 staining. D MSH6 negative in tumour cells. E Positive PMS2 staining. F Real-time PCR (ARMS sequencing) showed G13D mutation in K-ras. G Sanger sequencing showed 602 G>A mutation in GNAS.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable5.7.doc