Molecular alterations in colitis-associated colorectal neoplasia: Study from a low prevalence area using magnifying chromo colonoscopy

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KEYWORDS
Ulcerative colitis; Colorectal neoplasia; Magnifying chromo colonoscopy; Microsatellite instability; p53

Abstract

Background and aim: Longstanding ulcerative colitis (UC) predisposes to colorectal cancer (CRC). To understand the molecular pathogenesis of colitis-associated colorectal neoplasia (UC-CRN), we studied the frequency of microsatellite instability (MSI) and mutations in p53, BRAF and KRAS genes in the tissues of patients with long standing UC with or without neoplasia and compared them with colitis patients without risk of neoplasia, and those with sporadic colorectal neoplasia (S-CRN) in an area with lower prevalence for either disease.

Methods: Biopsies were obtained during magnifying chromo colonoscopy or routine colonoscopy in consecutive UC patients with high risk (UC-HR) and low risk (UC-LR) of neoplasia, and those with S-CRN. MSI (NCI-Bethesda panel) and mutations in p53, KRAS and BRAF genes were analysed.

Results: Twenty-eight patients with UC-HR, 30 with UC-LR and 30 with S-CRN were included. Six (21.4%) of UC-HR had neoplasia (Progressors). MSI was not detected in the UC-CRN group as compared to 5 (16.7%) in the S-CRN group. Mutations in p53 occurred in 6 (27.3%, P<0.05) and 3 (50%, P<0.05) in the UC-HR subgroups without and with neoplasia respectively, as against 10 (33.3%) in sporadic neoplasia group. KRAS mutations were found only in the presence of neoplasia. None showed the BRAF mutation.

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1. Introduction

Ulcerative colitis (UC) and sporadic colorectal neoplasia (S-CRN), both diseases of the Western world, are being increasingly reported from many Asian countries. Long-standing ulcerative colitis increases the risk of development of colorectal cancer (CRC), the life-time risk being 3.7% increasing to as high as 18%, 30 years after the onset of the disease. The risk of CRC in UC has been variably reported from different parts of Asia. While several environmental and genetic factors contribute to the development of S-CRN, chronic inflammation is thought to be additionally important in carcinogenesis in UC. S-CRN results from sequential alterations in genes that regulate the growth of colonic epithelial cells. On the other hand, ulcerative colitis-associated colorectal neoplasia (UC-CRN) progresses from inflamed epithelium to dysplasia to carcinoma consequent to episodic somatic mutations. Compared to sporadic neoplasm, UC-CRN shows characteristic clinicopathological features, different topographic distribution along the colon and a delay in diagnosis. This suggests that genetic alterations in UC-CRN might not be the same as those reported in S-CRN. Major gastrointestinal societies have published recommendations for the early detection of neoplasia in patients with UC using endoscopic surveillance. When compared to conventional white light endoscopy, chromo colonoscopy combined with magnification for classification of pit pattern has a higher diagnostic accuracy for distinguishing neoplasia.

The molecular changes that appear during adenoma–carcinoma sequence have been well studied in S-CRN. The two main pathways of tumour progression in S-CRN and UC-CRN, microsatellite instability (MSI) and chromosomal instability (CIN), are prevalent in about 15% and 85% respectively. MSI results from a failure in the proofreading mechanism of DNA replication. Approximately 15–20% of S-CRN and 15–40% in UC-CRN arise through the MSI pathway. MSI mainly involves a loss of function of genes that normally repair the mismatches between DNA base pairs that occur during the DNA replication in dividing healthy cells. Several DNA mismatch repair (MMR) genes play a major role in repairing these mismatches, which are often induced by oxidative stress accompanying chronic inflammation.

\( \text{K\textsc{ras}} \) is a proto-oncogene, actively involved in G protein-mediated signal transduction pathways and plays an important constitutive role with GTPase activity. Frequent mutations are observed in codons 12, 13 and 61 resulting in the increased and unregulated cellular proliferation. Several studies have reported the frequency of \( \text{K\textsc{ras}} \) mutations in UC-CRN to be 8–24% which is lower than that reported for sporadic neoplasms (40–50%). Mutations in \( \text{B\textsc{raf}} \) have been described in about 15% of all human tumours, especially in malignant melanomas. A single-base substitution (T1796A) resulting in an amino acid substitution (V600E) accounts for 80–90% of these mutations. \( \text{p\textsc{53}} \) encodes a major tumour suppressor protein that acts as a guardian of the genome, arresting the cell cycle in the G1 phase when DNA is damaged. Structurally human \( \text{p\textsc{53}} \), encoded by a 20 Kb gene, is a nuclear phosphoprotein of molecular weight 53 kDa, where amino acid residues within the central DNA-binding domain are found to be frequently mutated in human tumour tissues. Mutations in these codons account for approximately 43% of all \( \text{p\textsc{53}} \) mutations in CRC. There is considerable evidence for the role of \( \text{p\textsc{53}} \) in UC carcinogenesis starting from detection of mutations in early stage and increasing in frequency as the disease progresses from no dysplasia (6%) through low-grade and high-grade dysplasia towards cancer (85%).

Even in developed countries with higher prevalence of these diseases, surveillance colonoscopy remains unsatisfactory and impractical for all UC patients with conventional risk factors due to difficulties with endoscopic and histological diagnosis of UC-associated neoplasia. At this point of time, there is no recommendation from gastroenterological societies for the use of any biomarker in the evaluation of risk of dysplasia or CRC in patients with IBD. Establishing the molecular signatures of early neoplasia, could lead to studies on the use of these for their early detection. Our study aimed at evaluating the molecular changes that occur in UC at different stages of evolution to cancer by classifying the neoplasia using magnifying chromo colonoscopy in a population with low propensity to CRN. The presence of commonly described molecular alterations in both UC-CRN and S-CRN were compared. UC patients with low risk of neoplasia (UC-LR) served as negative controls.

2. Materials and methods

2.1. Subjects

Twenty-eight patients with UC at risk of associated colorectal neoplasia (≥7 years of extensive colitis or ≥10 years of left-sided colitis) underwent screening magnification chromo colonoscopy as a part of a surveillance programme from September 2008 to January 2011. They were subdivided as nonprogressors or progressors depending on the absence or presence respectively, of neoplastic lesions on histopathology. Thirty patients each with S-CRN (\( n=22 \) with adenocarcinoma, \( n=8 \) with adenoma) and UC-LR (\( n=30 \), duration of disease less than 7 years) seen during the same period were enrolled for comparison.

Those with S-CRN and UC-LR underwent endoscopic biopsies from affected and normal areas for histology and molecular analysis. The diagnosis of both UC and CRN was made according to established criteria, including clinical symptoms, colonoscopy and histopathology. Human colorectal cancer cell lines COLO 205, HT-29 and HCT-15 were obtained from National Centre for Cell Science (NCCS, Pune, Maharashtra, India) and...
DNA extracted from them was used as positive or negative controls for all molecular analyses, based on their mutation status as reported in the National Cancer Institute’s database (NCI-60).

This study was approved by the ethics committee of Kas turba Hospital, Manipal. All the patients provided informed consent before participation.

2.2. Magnifying chromo colonoscopy

Magnifying chromo colonoscopy was performed when the patients were in remission, as described previously, using an 80× magnification colonoscope (EC-3870 L2K, Pentax Corporations, Tokyo, Japan). The colon was prepared using Polyethylene Glycol electrolyte solution. Biopsies were obtained from the normal caecum, or if this segment was involved by UC, from the distal ileum for histology and MSI. The entire colon was evaluated, washing off debris and mucus as necessary. After an initial overview of a segment, 10–20 ml of 0.4% indigo carmine (Sigma-Aldrich, USA) was sprayed, excess dye suctioned and all suspicious or raised areas were evaluated in detail under magnification for the pit patterns as described previously. Biopsies were obtained in pairs from closely adjacent sites from the areas of abnormal pit pattern, one bit from each pair being assigned for histopathology and the other for molecular analysis. If there were few or no areas of abnormal mucosal pattern, 4 quadrant biopsies were obtained every 10 cm and additionally from pseudo-polyps or raised areas aiming for a total of 30–40 biopsies. Patchy areas of disease activity, if found, were avoided as far as possible for tissue sampling. The biopsies were classified according to Riddell histological grading as active disease, UC in remission, dysplasia [indefinite, low grade (LGD) or high grade (HGD)] or cancer. UC-HR patients were further classified as progressors and nonprogressors depending on whether neoplastic lesions were present or not, respectively.

2.3. DNA extraction

Fresh biopsy specimens were immediately digested with proteinase K (0.1 mg/ml) in the presence of 1% Sodium Dodecyl Sulphate (Sigma-Aldrich, USA). DNA was extracted using phenol–chloroform, followed by ethanol precipitation. DNA was checked for purity and stored at −20 °C until further analysis.

2.4. Multiplex PCR and microsatellite analyses

Microsatellite instability (MSI) status was examined using 5 microsatellite markers (National Cancer Institute, Bethesda Panel). The primer sequences and the corresponding fluorescent dyes are listed in Table 1. Multiplex PCR was performed using Veriti thermocycler (Applied Biosystems, Foster City, CA) using the following cycling conditions: 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final 45 min, 60 °C extension to aid non-template adenine addition. The PCR products were analyzed using ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) along with GS500LIZ size standard according to the manufacturer’s instructions. The generated data were analyzed using Genemapper v.4.0 (Applied Biosystems, Foster City, CA). The samples were graded as MSI-Low or MSI-High depending on the number of peak shifts or abnormal alleles in the microsatellite loci when compared with the normal DNA from the same patient.

2.5. Mutation analysis of p53 and KRAS gene by PCR-sequencing

The central DNA-binding core domain encompassing exons 4, 5–6, and 7–8 of p53 gene and the first exon of KRAS were amplified by PCR using primers as listed in Table 2. The following conditions were used: (for p53 amplification) 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min and (for KRAS amplification) 95 °C for 5 min, followed by 34 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Obtained PCR products were purified using the MN-Nucleospin extract-II PCR purification kit (Macherey-Nagel, GmbH, Germany) and the resulting samples were directly sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The presence and nature of each mutation was confirmed by repeat PCR and sequencing.

<table>
<thead>
<tr>
<th>Marker (gene)</th>
<th>Chromosomal location (gene)</th>
<th>Microsatellite repeats</th>
<th>Primer sequence (from 5′ to 3′)</th>
<th>Product length (in base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT25</td>
<td>4q12 (c-kit)</td>
<td>A(25)</td>
<td>F: NED TGCCTCAAGAATGTAAGT</td>
<td>110–130</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TCTGCTTTAAAATGATGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V: VIC TCACTCTTTGATCTCTCCC</td>
<td>100–120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: AAGGCTAAATTTTAAACC</td>
<td></td>
</tr>
<tr>
<td>BAT26</td>
<td>2p16.3-p21 (hMSH2)</td>
<td>A(26)</td>
<td>F: NED AAGAAGAAGGTGCTCTTTTA</td>
<td>197–227</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GGACCTCTACCATATGGGAC</td>
<td></td>
</tr>
<tr>
<td>D2S123</td>
<td>2p16 (hMSH2)</td>
<td>CA(n)</td>
<td>F: FAM ACTCTACTGTTAATACG</td>
<td>96–122</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: AGAGATTGAGAGCTATTGTTG</td>
<td></td>
</tr>
<tr>
<td>D5S346</td>
<td>5q21-22 (APC)</td>
<td>CA(n)</td>
<td>F: FAM GAGAGGCTCTTATATATAT</td>
<td>151–169</td>
</tr>
<tr>
<td>D17S250</td>
<td>17q11.2-12 (p53/Mfd15CA)</td>
<td>CA(n)</td>
<td>F: GAGAGGCTCTTATATATATATAT</td>
<td></td>
</tr>
</tbody>
</table>

Forward primers modified with particular dyes (VIC, FAM, NED) feasible for ABI 3130 Genetic Analyzer (G5 panel set).
2.6. **Analysis of** **BRAF** T1796A mutation by PCR-RFLP

PCR was performed using **BRAF** exon 15 specific primers (Table 2), as described elsewhere.39 PCR amplification was done in Eppendorf thermocycler (Eppendorf, Hamburg, Germany) using the following conditions: 95 °C for 5 min, followed by 34 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, followed by a final 10 min, 72 °C extension step. The 224 bp amplified products were subjected to Restriction Fragment Length Polymorphism (RFLP) analysis as described previously.39 Briefly PCR product was incubated with TspRI restriction enzyme (10 U/μl New England Biolabs, Beverly, MA) in a 20 μl reaction volume overnight at 65 °C, followed by running it on a non-denaturing 10% polyacrylamide gel. Random samples along with positive cell line products were sequenced for reconfirmation of the results.

2.7. **Statistical analysis**

Comparison of mutational status among the various histological groups was done using Fisher’s exact test and χ²-test as appropriate, in GraphPAD InStat (California, USA) software. The accepted statistical significance level was defined as \( P < 0.05 \).

3. **Results**

In the UC-HR group comprising of 21 men and 7 women aged 26–60 (median = 43.5) years, 17 had extensive colitis and 11 had limited colitis. The duration of UC ranged from 7 to 22 (median = 10) years. None had sclerosing cholangitis or a family history of colorectal cancer. Three (10.7%) of the men and none of the women were smokers. Six (21.4%) patients were classified as progressors, of whom 2 had LGD (1 in flat mucosa, 1 in adenoma-like polyp), 3 had HGD (2 in sessile polyps, 1 in a flat ulcer) and 1 had adenocarcinoma (sessile growth). During surveillance colonoscopy, endoscopic remission was seen in 15 (53.6%) patients, a few patches of mild and moderate disease in 10 (35.7%) and 3 (10.7%) respectively and severe endoscopic disease in none. Among the patients detected to have neoplasia, the endoscopic activity was normal in 4, mild in 2 and moderate in none. The UC-LR and S-CRN groups were comprised of 30 patients each with an age range of 19–82 (median = 37) years, and 26–88 (median = 61) years respectively (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Patient group (Sample size)</th>
<th>Neoplastic lesions</th>
<th>Male: female</th>
<th>Age (Median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC-LR (n = 30)</td>
<td>Nil</td>
<td>17:13</td>
<td>37 (19–82)</td>
</tr>
<tr>
<td>UC-HR (n = 28)</td>
<td>Nonprogressors, n = 22</td>
<td>21:7</td>
<td>43.5 (26–60)</td>
</tr>
<tr>
<td></td>
<td>Progressors n = 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-CRN (n = 30)</td>
<td>Adenomas n = 8</td>
<td>18:12</td>
<td>61 (26–88)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma n = 22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

UC patients grouped as low-risk (UC-LR) and high-risk (UC-HR). Progressors are patients with dysplasia or carcinoma; nonprogressors are patients without any neoplasia. Sporadic colorectal neoplasia (S-CRN).
3.2. Mutational analyses

Mutations in p53 were detected in 10 (45.5%) of the 22 samples with adenocarcinoma as against none with adenomas in the S-CRN group ($P < 0.05$). The frequency of these mutations was higher in UC-HR nonprogressors (6/22, 27.3%, $P < 0.05$) and UC-HR progressors (3/6, 50%, $P < 0.05$) compared to those with UC-LR (1/30, 3.3%) (Table 4). The mutations were distributed across exons 4–8 and more prevalent in exons 7–8 and exons 5–6 than in exon 4. Two heterozygous insertions or deletions were also observed in exon 4 and exons 5–6, in the sporadic adenocarcinoma group. In the cell lines, COLO 205 had 24 bp deletions in exon 4 whereas the other two cell lines had a single mutation each in exons 7–8.

The presence of mutations in codons 12 and 13 of KRAS was determined. In the S-CRN group, 2 (25%) of the 8 adenomas and 4 (18.2%, $P > 0.05$) of the 22 adenocarcinomas showed mutation (Table 4). Of these, three had G13D (GGC-GAC), two had G12D (GGT-GAT) and one had G12C (GGT-TGT) mutations. None of the UC-LR samples showed mutant KRAS. In case of UC-HR, nonprogressors showed wild type of KRAS, but in 1 of 5 progressors (20%) a G13D mutation was observed; this patient had high grade dysplasia. Among the cell lines analyzed only HCT-15 showed KRAS G13D mutation.

None of the samples analyzed from UC-HR, UC-LR or S-CRN groups, showed the T1796A (V600E) mutation in exon 15 of BRAF gene. HT-29 and COLO 205 were positive and HCT-15 was negative for the mutation analyzed.

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Table 4  Comparison of molecular alterations in p53, KRAS and BRAF genes and MSI in all three different study groups and subgroups.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Neoplastic lesions</th>
<th>MSI</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No (%)</td>
</tr>
<tr>
<td>UC-LR (n=30)</td>
<td>Nil</td>
<td>0</td>
<td>1 (3.3%)</td>
</tr>
<tr>
<td>UC-HR (n=28)</td>
<td>Nonprogressors</td>
<td>0</td>
<td>6 (27.3%)a</td>
</tr>
<tr>
<td></td>
<td>Progressors</td>
<td>0</td>
<td>3 (50%)b</td>
</tr>
<tr>
<td>S-CRN (n=30)</td>
<td>Adenomas</td>
<td>0</td>
<td>2 (25%)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>5 (22.7%)c</td>
<td>10 (45.5%)</td>
</tr>
</tbody>
</table>

a $P < 0.05$ when comparing p53 gene alterations of UC-HR nonprogressor with UC-LR.
b $P < 0.05$ when comparing p53 gene alterations of UC-HR progressor with UC-HR nonprogressor and UC-LR.
c $P = 0.052$ showing comparison of MSI in S-CRN vs. UC-LR and UC-HR.

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Figure 1  Microsatellite Instability (MSI) analysis for Bethesda panel of markers: A) multiplex PCR products of normal (Nr) and abnormal (Ab) on agarose gel along with marker (M); B) Matching normal (Nr) and abnormal sample (Ab) analyzed through the capillary electrophoresis and fragment analysis showing the presence of instability in the abnormal sample.
None of the tested mutations were observed in the single patient with UC-HR displayed mutations in both p53 and KRAS as a raised targeted area and was pathologically graded as high grade dysplasia, with a significant higher frequency of mutations in the later stages of progression. In contrast, the S-CRN group showed a raised targeted area and that they occurred increasingly commonly in the progres- sion to CRC, with a steady increase in frequency among biopsies that showed progressive degrees of dysplasia. The frequency of p53 mutation in non-dysplastic UC tissues still remains controversial. A previous study using tissues from UC patients without cancer, demonstrated a high frequency of p53 mutations in the inflamed mucosa, suggesting that chronic inflammation may predispose to these changes.

Loss of heterozygosity and mutations in p53 occur late in the pathogenesis of S-CRN but is an early event in the progression to UC-CRN.

In the progressor group 4 out of 5 samples showed mutations in p53 (n=3) or KRAS (n=1). One sample that showed a raised targeted area and was pathologically graded as HGD displayed mutations in both p53 and KRAS (Fig. 2). None of the tested mutations were observed in the single patient with colitis cancer.

4. Discussion

By classifying patients with UC-HR using magnification chromo colonoscopy to stratify disease progression in UC and molecular analysis for early detection and comparing two different pathways of colorectal carcinogenesis.

MSI is believed to play a role in both the pathways of UC-associated and sporadic CRC, as it is reported to be slightly more frequent in the former than the latter. Despite previous studies reporting the presence of MSI in UC (with a frequency ranging between <1% and 70% in UC progression to CRC), both in non-neoplastic and neoplastic conditions, our study using NCI panel of Bethesda markers found no instability in UC-LR or UC-HR samples.

Loss of heterozygosity and mutations in p53 occur late in the pathogenesis of S-CRN but is an early event in the progression to UC-CRN. Mutations of p53 occur in 33–100% of UC-associated CRN and 75–80% in S-CRN. However, confirmed mutations occurred in 19% of UC biopsies without dysplasia, with a steady increase in frequency among biopsies that showed progressive degrees of dysplasia.

The frequency of p53 mutation in non-dysplastic UC tissues still remains controversial. A previous study using tissues from UC patients without cancer, demonstrated a high frequency of p53 mutations in the inflamed mucosa, suggesting that chronic inflammation may predispose to these changes.

In our study, p53 mutations were found in 3.3% of UC-LR, increasing to 27.3%, (P<0.05) and 50%, (P<0.05) in the UC-HR subgroups without and with neoplasia respectively, as against 45.5% only in adenocarcinoma of S-CRN. These mutations were not specific for any particular codon and were spread across the region spanning exons 4 to 8.

KRAS and BRAF oncogenes are involved in the MAP-kinase pathway and activate cellular growth through different mechanisms. KRAS mutations are often found in the premalignant stages of its pathogenesis. In UC-CRN, KRAS mutations seem to arise late in the development of the disease and with low frequency. Frequencies of reported mutation vary between 5 and 50%, which are comparable to those of S-CRN. In comparison, we found KRAS mutations in one out of six (16.7%, P>0.05) patients with UC-associated high grade dysplasia and in 25% and 18.2% of sporadic colorectal adenomas and adenocarcinomas respectively. Previous studies have described these mutations in none of the longstanding UC or LGD, 7% with HGD and 25% with CRC. Based on available evidence it is difficult to assess the role of KRAS mutations in the development of cancer.

BRAF mutations, in general, have been reported less frequently in sporadic colorectal cancers (6–11%) and in UC-associated neoplasia (9%). There is increasing evidence that BRAF mutations in colorectal cancers are more frequent in microsatellite unstable tumours that develop through the so-called mutator or serrated pathway. However, no BRAF T1796A alteration was found in the samples analyzed.
The small number of patients in our study, especially in the subgroups of interest, is an obvious limitation. However, coming as it does from an area with a low prevalence of both UC and CRC, it is not surprising that the numbers studied were small. Even in areas with high prevalence of the two diseases, adherence to surveillance is suboptimal and consequently the numbers of neoplasia picked up in patients with UC is not very high.17,47,48

In summary, in a population with a lower prevalence of UC and CRC, the present findings, using magnification chromo colonoscopy and targeting abnormal areas, show that the molecular changes in selected genes involved in the colorectal carcinogenesis with and without underlying UC differ. These changes, except for the frequency of MSI, are similar to those described in populations with a higher prevalence for either disease, which opens up the possibility for a better understanding of the problem universally. To overcome the limitations and to improve the efficacy of early detection of neoplasia, future studies could assess the role of surveillance with advanced imaging combined with newer molecular markers in the pathogenesis of CRC.

Conflict of interest
All the authors declare no conflicts of interest.

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KS and CGP conceived the project and along with BMSK and BLK designed the study, BMSK and BLK collected samples and clinical data, BMSK and BLK performed the molecular experiments, BMSK analysed the data, CGP, GB and DS did the clinical and endoscopic evaluation, LR performed all experiments, BMSK analysed the data, CGP, GB and DS did pathological characterizations and KS provided support for the clinical and endoscopic evaluation, LR performed all experiments, BMSK and BLK collected samples and BLK designed the study, BMSK and BLK collected samples and clinical data, BMSK and BLK performed the molecular experiments, BMSK analysed the data, CGP, GB and DS did the clinical and endoscopic evaluation, LR performed all experiments, BMSK and BLK collected samples and BLK designed the study.

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