SPANDIDOS PUBLICATIONS

Involvement of the serrated neoplasia pathway in inflammatory bowel disease-related colorectal oncogenesis

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Abstract. The purpose of this study is to identify colorectal serrated lesions in the inflammatory mucosa of inflammatory bowel disease (IBD), to characterize their molecular status based on *BRAF* and *KRAS* mutations, mismatch-repair (MMR) deficiency and microsatellite instability (MSI), and to verify that these molecular alterations are specific to the 'serrated neoplasia pathway' in IBD. Neoplastic lesions from 36 patients with IBD were reviewed retrospectively, including 13 adenocarcinomas (1 mucinous and 12 conventional), 28 dysplasias [1 traditional serrated adenoma (TSA) and 27 conventional adenomas] and 1 hyperplastic polyp (HP). Both the HP and TSA exhibited the V600E *BRAF* mutation without MSI or MMR deficiency. The mucinous adenocarcinoma, close to the TSA, exhibited the *BRAF* mutation and MSI with loss of hMLH1. No *KRAS* mutations were found in these 3 lesions, and no *BRAF* mutations were found in the conventional ones. Serrated lesions exist in the inflammatory mucosa of IBD and are associated with a characteristic molecular profile, i.e. the appearance of the *BRAF* mutation as early as the hyperplastic polyp stage followed by MSI at the carcinoma stage. We therefore identified the serrated neoplasia pathway in IBD-related colorectal oncogenesis.

Introduction

Patients with ulcerative colitis (UC) and Crohn's disease (CD) are at an increased risk of developing colorectal cancer (CRC) through an inflammatory/regenerative/dysplasia/carcinoma sequence (1,2). Clinical factors favouring CRC include the duration and the extent of the inflammatory bowel disease (IBD) (3). They are in part responsible for the development of sequential or synchronous multiple neoplastic lesions throughout the colonic mucosa, which require regular colonoscopic surveillance. IBD-associated CRC arises from dysplastic precursor lesions such as dysplasia-associated lesion or mass (DALM) or dysplasia in flat mucosa (4).

Recently in sporadic CRCs, a novel oncogenic pathway - the 'serrated neoplasia pathway' - has been defined by its epidemiological, morphological and molecular characteristics. In fact, carcinomas of this serrated neoplasia pathway represent a distinctive group of colorectal neoplasms that show defective DNA mismatch-repair (MMR) which results in microsatellite instability (MSI) (5). The other molecular characteristics of these cancers are a strong association with activating mutations of *BRAF* and a negative association with *KRAS* mutations (5-7). The penultimate stage in the progression to carcinoma of this pathway are serrated polyps (8) including hyperplastic polyps (HP), traditional serrated adenomas (TSA) and the more recently described sessile serrated adenomas (SSA). Furthermore, there is evidence from many studies that some categories of serrated polyps previously classified as HPs are indeed the precursors of serrated adenoma (5,8-10). In addition, recent findings suggest that the *BRAF* V600E mutation is a specific marker for the serrated pathway originating from an HP (8).

Until now, there has been no mention of serrated lesions in studies focused on preneoplastic lesions in the inflammatory mucosa in IBD. In order to assess the involvement of the so-called serrated neoplasia pathway in IBD-related oncogenesis, we retrospectively reviewed a cohort of patients with IBD-associated preneoplastic and neoplastic lesions. The purpose of this study was threefold. First, we retrospectively reviewed all samples of preneoplastic and neoplastic lesions to identify morphologically serrated lesions in the inflammatory mucosa. Second, we attempted to characterize the molecular status of these lesions based on the determination of *BRAF* and *KRAS* mutations, the MMR deficiency status as assessed by immunohistochemistry and the MSI status. Finally, we attempted to verify the specificity of these molecular alterations to the serrated neoplasia pathway.

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Patients and methods

Patients and tissues. A series of 91 samples from a cohort of 36 patients with IBD-related neoplasias (32 UC, 4 CD) were reviewed retrospectively. These patients had no familial history of CRCs. We selected only lesions located in the inflammatory mucosa of the colon or rectum. For each patient, matching inflammatory mucosa was collected, when possible, and served as an internal control. Additionally, 22 samples from the inflammatory mucosa of 22 patients with IBD without neoplasia served as controls (20 UC and 2 CD).

Fixed and paraffin-embedded tissue specimens, obtained from surgical resection or biopsies performed between 1993-2006, were collected from the archives of the Departments of Pathology of the Centres Hospitaliers Universitaires of Nantes, Rennes and Poitiers (France), of the Institut d'Histopathologie (Nantes, France) and of the Centre Hospitalier Départemental (La Roche sur Yon, France), and were processed according to the guidelines of the French Ethics Committee for Research on human tissues.

Methods

Histological classification. Classification and grading of carcinomas, dysplasias and indefinite lesions for dysplasia were performed on hematoxylin, eosin and safran (HES) stained sections according to standardized histological consensus criteria (2). Briefly, atypical changes were classified into 3 distinct categories: negative, indefinite and positive for dysplasia. Dysplastic mucosae were further subclassified as low- and high-grade depending on the severity of the atypia. Serrated lesions were subclassified according to the schema of Torlakovic *et al* (11). Among carcinomas, tumor typing was performed according to WHO criteria (12).

Genomic DNA extraction. Genomic DNA was extracted using the Qiagen tissue DNA kit (Qiagen, Courtaboeuf, France). The extractions were taken from paraffin-embedded (10 μm thick) sections of the 91 samples and 22 controls. For each neoplastic sample, the histopathologic region of interest was identified on a HES-stained section and a manual microdissection was performed to isolate the neoplastic cell populations.

BRAF and KRAS mutation analysis. Evaluation of the *BRAF* codon 600 mutation as well as the *KRAS* exon 1, codons 12 and 13 mutations was performed by a quantitative allele-specific polymerase chain reaction (PCR) designed and validated previously in our laboratory (13,14). PCR primers for the hotspot region of the *BRAF* gene on exon 15 (T1799A) were used.

MSI status. The MSI-H status was determined by PCR to amplify 5 mononucleotide markers, BAT25, BAT26, NR21, NR22 and NR24, designed and validated previously (15). Briefly, the 5 mononucleotide repeats were coamplified in a single pentaplex PCR reaction. The pentaplex PCR was performed under the following conditions: denaturation at 94˚C for 2 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 57˚C for 60 sec and extension at 72˚C for 60 sec. This was followed by an extension step at 72˚C for 30 min. PCR products were analysed on an ABI PRISM 3100 capillary automated DNA sequencer. Genscan software (Genotyper 2.1, Applera, France) was used to calculate the size of each fluorescent PCR product.

Lesions were characterized as MSI-H if they manifested instability at 2 or more loci, or microsatellite stable (MSS) if they showed no instability at any locus.

Immunohistochemistry of MMR proteins hMLH1, hMSH2 and hMSH6. For each case, 3 μm thick sections were stained using a standard 3-step streptavidin-biotin-peroxidase complex method with 3,3-diaminobenzidine as chromogen (ChemMate® Streptavidin Peroxidase Kit, Dako, Trappes, France). The following antibodies were used in fixed and deparaffinised tissue sections: hMLH1 (G168-15, BD Biosciences, Erembodegem, Belgium), hMSH2 (D06571-8, Oncogene, Cambridge, MA, USA) and hMSH6 (2D4B5, Zymed). All antibodies required antigen retrieval in a boiling citrate buffer.

Lesions showing nuclear immunostaining for hMLH1, hMSH2 or hMSH6, whatever the intensity and the percentage of epithelial neoplastic nuclei stained, were considered as expressing these MMR proteins.

Statistical correlations. Statistical analyses were performed with GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA). Fisher's exact method was used to test the associations between mutational status, MMR deficiency and clinicopathologic features. A two-tailed probability of 0.05 was accepted as statistically significant.

Results

Histopathological characteristics of lesions. Each sample was examined by two pathologists (C.B. and J.F.M.) and lesions were reclassified according to the Riddell classification (2). Serrated polyps were classified using the system described by Torlakovic *et al* (11). All lesions were located in the inflammatory mucosa of IBD.

Among the 13 adenocarcinomas, there were 12 well to moderately differentiated adenocarcinomas (right colon, n=6; left colon, n=5; transverse colon, n=1), 1 mucinous adenocarcinoma of the right colon and 1 signet-ring cell carcinoma of the left colon. Among the 8 high-grade dysplasias, 3 developed from flat mucosa in the left colon, while 5 corresponded to sessile or pedonculated polyps (left colon, n=1; right colon, n=2, transverse colon, n=1; unknown location, n=1) considered as DALM. All corresponded to conventional adenomas. Among the 20 low-grade dysplasias, 4 developed from flat mucosa (left colon, n=3; right colon, n=1) and 16 were sessile or pedonculated polyps, or DALM (left colon, n=10; right colon, n=5; transverse colon, n=1). Nineteen corresponded to conventional adenomas while 1 sessile polyp of the right colon, close to the mucinous adenocarcinoma, corresponded to a TSA with low-grade dysplasia. In addition, we found 1 HP with microvesicular cells (microvesicular cell type hyperplastic polyp) developed in the caecum. Lastly, serrated lesions (HP and SSA) accounted for 6.9% of the preneoplastic lesions in the inflammatory mucosa. The 6 indefinite lesions for dysplasia and 8 regenerative mucosae were also analysed.

Molecular alterations of serrated lesions.

	BRAF mutation	*KRAS* mutation	Microsatellite status	Immunohistochemistry		
				hMLH1	hMSH2	hMSH6
Hyperplastic polyp	+	-	MSS	+	+	+
Traditional serrated adenoma	+	-	MSS	+	+	+
Mucinous adenocarcinoma	+	-	MSI	-	+	+

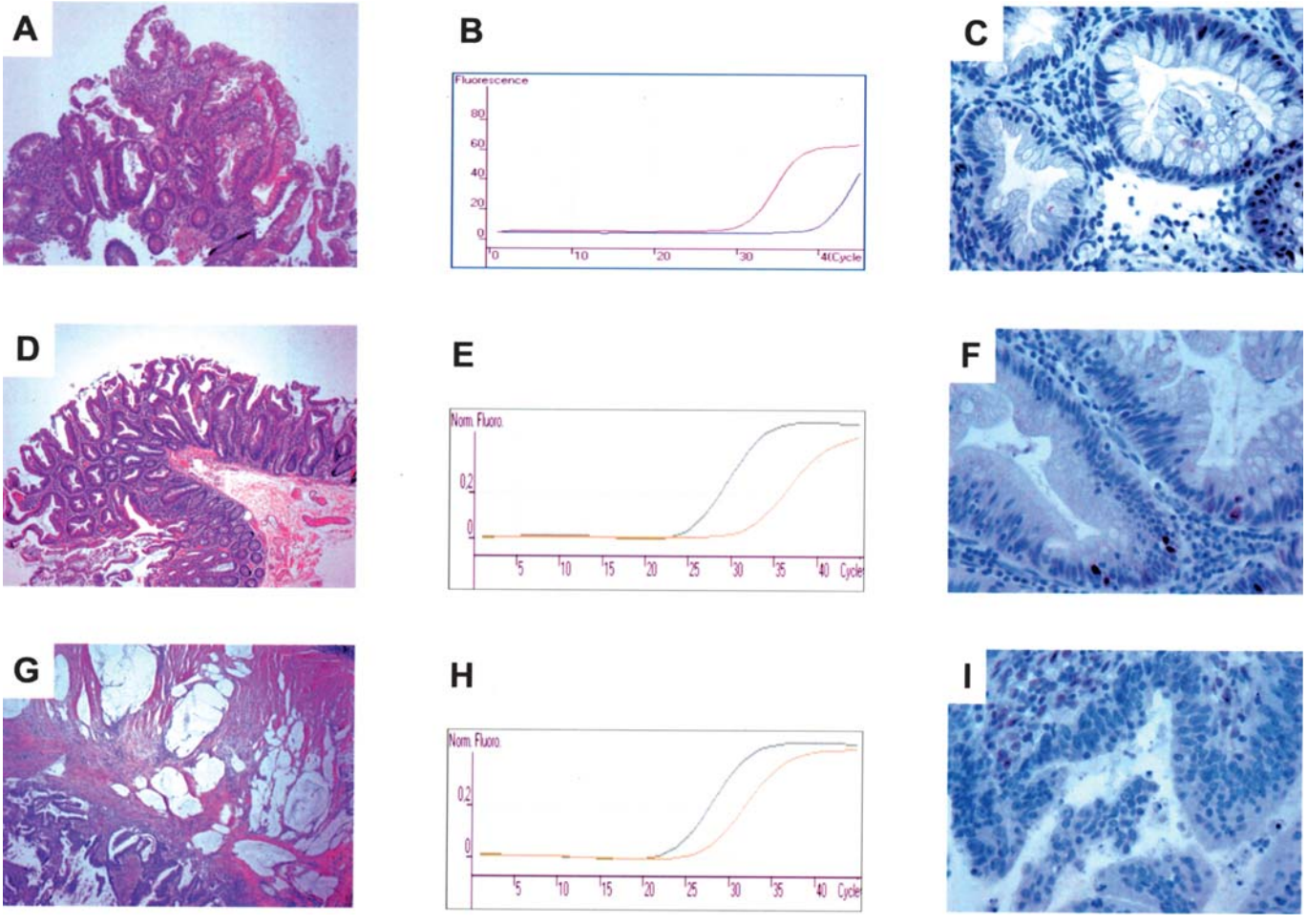

Figure 1. Molecular profile of morphological serrated lesions in IBD. (A-C) A hyperplastic polyp of the caecum, microvesicular type. (A) Morphological features (hematein, eosin and safran stain, x10): serrated architecture, mainly microvesicular with few goblet cells, no dysplasia. (B) V600E BRAF mutation detected by quantitative allele-specific polymerase chain reaction (fluorescence curves). (C) Immunohistochemistry for hMLH1 protein showing positive staining of many epithelial nuclei (x40). (D-F) A traditional serrated adenoma with low-grade dysplasia. (D) Morphological features (hematein, eosin and safran stain, x10): villiform configuration of the lesion, relatively uniform epithelium lining the polyp consisting of eosinophilic cells with central focally pseudostratified nuclei. (E) V600E BRAF mutation detected by quantitative allele-specific polymerase chain reaction (fluorescence curves). (F) Immunohistochemistry for hMLH1 protein showing positive staining of many epithelial nuclei (x40). (G-I) A mucinous adenocarcinoma of the right colon. (G) Morphological features (hematein, safran and eosin stain, x10): pools of extracellular mucin contain malignant strip of epithelial cells. (H) V600E BRAF mutation detected by a quantitative allele-specific polymerase chain reaction (fluorescence curves). (I) Immunohistochemistry for hMLH1 protein showing loss of expression by all tumor cells (x40).

Molecular status of the serrated lesions. Genomic DNAs were tested for mutations in exon 15, codon 600 of the *BRAF* gene and in exon 1, codons 12 and 13 of the *KRAS* gene.

Table I shows that both the HP and the TSA exhibited the V600E *BRAF* mutation without MSI or loss of MMR proteins expression (Fig. 1A-F). Interestingly, the mucinous adenocarcinoma of the right colon close to the TSA exhibited, by immunohistochemistry, both the *BRAF* mutation and MSI-H status with loss of hMLH1 (Fig. 1G-I). No *KRAS* mutations were found in these 3 particular lesions. The

corresponding non-neoplastic inflammatory mucosa of these lesions had a wild-type *BRAF* gene, MSS status and expression of the 3 MMR proteins.

BRAF mutation is specific to serrated lesions in IBD. The *BRAF* mutation was not found in conventional adenomas or adenocarcinomas, or in the inflammatory mucosae, regenerative mucosae or indefinite lesion for dysplasia. *KRAS* mutations (n=8) in either codon 12 or 13 of exon 1 were found in 7 of the 91 samples (7.7%). Two mutations (12GA and 12GT) were found in the same sample, concerning a flat low-grade dysplastic area of the right colon. Seven of the 8 mutations (87.5%) were in codon 12, most often in the second position, leading to the substitution of a guanidine to an adenine (G→A) or a thymidine (G→T). One mutation was observed in the second position in codon 13. These *KRAS* mutations were detected in 1/13 adenocarcinomas (7.7%), 5/28 dysplasias (17.8%; low-grade, n=3; high-grade, n=1) and 1/34 inflammatory mucosae (2.9%). One *KRAS* mutation in codon 12 was detected in inflammatory mucosa among the 22 controls (4.5%). Mutations were statistically more frequent in dysplasias and cancers than in regenerative or inflammatory mucosae (Fisher's exact test, p=0.047). All IBD-related neoplasias with a *KRAS* mutation had a wild-type *BRAF* gene.

Discussion

To the best of our knowledge, this is the first report on the individualisation of serrated lesions in inflammatory mucosa in relation to their molecular profile across the entire histological spectrum of the so-called serrated neoplasia pathway in IBD. The main findings of this retrospective study are threefold. First, the existence of preneoplastic morphological serrated lesions, similar to those already described in sporadic colorectal oncogenesis, in the inflammatory mucosa of IBD. Second, that these morphological lesions are associated with a particular molecular profile. Finally, that these molecular alterations seem to be specific to serrated lesions.

Moreover, we report on the occurrence of serrated preneoplastic lesions located in inflammatory mucosa, accounting for 6.9% (2/29) of all preneoplastic lesions. This is a rather low percentage compared to the number of serrated lesions diagnosed in patients without IBD (16,17), but it probably corresponds to an underestimation of these lesions due in part to a sample size phenomenon. In addition, as HPs have traditionally been considered non-progressive lesions with no malignant potential (18) they are probably often ignored and not mentioned in pathological reports.

It is now well established in sporadic colorectal oncogenesis that the serrated neoplasia pathway describes a morphologic progression originating from an HP and terminating in advanced adenocarcinoma, which has pathologic, biologic and clinical characteristics that distinguish it from adenocarcinomas of the traditional adenoma-carcinoma sequence. In fact, a high frequency of activating *BRAF* mutation across the histological spectrum from HP to serrated adenoma has been previously reported (8,10,17,19), supporting the fact that *BRAF* mutations represent an early and instigating event in the serrated neoplasia pathway (10,19). As well, some authors have reported a link between *BRAF* mutated serrated polyps and the ultimate development of MSI sporadic colorectal carcinomas (5,8). Our present study illustrates the high sensitivity of the activating V600E *BRAF* mutation as a biomarker for the serrated neoplasia pathway in inflammatory mucosa in IBD. Furthermore, the occurrence of a mucinous adenocarcinoma of the right colon exhibiting a *BRAF* mutation and MSI-H status close to a TSA with a *BRAF* mutation and MSS status illustrates, in IBD-related oncogenesis, the fact that the *BRAF* mutation is an early molecular event preceding MSI, as in sporadic oncogenesis (8).

Interestingly, in our study the *BRAF* mutation is specific to the serrated neoplasia pathway in IBD-related oncogenesis. It was identified in all serrated preneoplastic lesions and in the MSI mucinous adenocarcinoma of the right colon, and was not found in any of the histological categories of the traditional adenoma-carcinoma sequence. These results in IBD-related colorectal oncogenesis are in line with the literature concerning sporadic colorectal oncogenesis (5,8,17). *KRAS* mutations are observed preferentially in these traditional lesions, as in sporadic colorectal oncogenesis (17,20), and have been intensively studied throughout the inflammatory/regenerative/dysplasia/cancer sequence in IBD (20-22). These activating mutations occur very early in IBD-related oncogenesis, since *KRAS* mutations are detected in control inflammatory mucosa devoid of dysplasia (2.8%). Additionally, our results, which show that *KRAS* mutations are more frequent than *BRAF* mutations in IBD-associated adenocarcinoma, are in line with a study by Aust *et al* which reports 18% of *KRAS* compared with 9% of *BRAF* mutations in UC-related adenocarcinomas (23).

In conclusion, we have demonstrated that the serrated neoplasia pathway can be involved in IBD-related colorectal oncogenesis. This observation has important practical consequences. In fact, such lesions, and particularly HPs, are probably overlooked. Pathologists must reassess their reporting practices with regard to HPs and SSAs in inflammatory mucosa, since they may be biomarkers for the future development of cancer. Likewise, endoscopists should view HPs with the same consideration as they would apply to conventional adenomas in patients with IBD.

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