Frequent mutations of KRAS in addition to BRAF in colorectal serrated adenocarcinoma

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Aims: To define the occurrence of KRAS and BRAF mutations, microsatellite instability (MSI), and MGMT and hMLH1 methylation and expression in colorectal serrated adenocarcinoma.

Methods and results: KRAS codon 12/13 and 59/61 and BRAF V600E mutations, MSI, and MGMT and hMLH1 methylation and expression in 42 serrated adenocarcinomas and 17 serrated adenomas were compared with those in 59 non-serrated colorectal carcinomas (CRCs) and nine adenomas. KRAS and BRAF mutations were observed in 45% and 33% of serrated adenocarcinomas and in 27% and 0% of non-serrated CRCs (P < 0.001). The KRAS c12G → A transition was the predominant type of mutation in serrated adenocarcinomas. Forty-two per cent of BRAF-mutated serrated adenocarcinomas showed high-level MSI (MSI-H) (P = 0.075), 100% showed hMLH1 methylation (P = 0.001) and 90.9% showed MGMT methylation (P = 0.019). Fifty-six per cent of serrated adenocarcinomas with microsatellite stability/low-level microsatellite instability harboured KRAS mutations. In non-serrated cancers, KRAS mutations were not associated with MSI status.

Conclusions: A high combined mutation rate (79–82%) of KRAS and BRAF in serrated adenomas and adenocarcinomas indicates that mitogen-activated protein kinase activation is a crucial part of the serrated pathway. BRAF mutations are specific for serrated adenocarcinoma and identify a subset of serrated adenocarcinomas with gene methylation and a tendency for MSI-H. A high frequency of KRAS mutations in serrated adenocarcinomas suggests that a significant proportion of KRAS-mutated CRCs originate from serrated precursors, thus challenging the traditional model of Vogelstein.

Keywords: BRAF, colorectal cancer, DNA hypermethylation, hMLH1, KRAS, MGMT, microsatellite instability, serrated adenocarcinoma

Abbreviations: CIM, CpG island hypermethylation; CRC, colorectal carcinoma; MAPK, mitogen-activated protein kinase; MSI, microsatellite instability; MSI-H, high-level microsatellite instability; MSI-L, low-level microsatellite instability; MSS, microsatellite stability; PCR, polymerase chain reaction

Introduction

Colorectal cancer (CRC) is the second most common cancer type in the Western world.1 For a long time, non-serrated adenomas were thought to represent the only significant precursor lesion for CRC.2 However, it is now apparent that the development of 15–20% of sporadic CRCs is not explained by Vogelstein’s adenoma–carcinoma model. These cancers often show concurrent BRAF mutations and DNA CpG island hypermethylation (CIM), and associate with high-level DNA microsatellite instability (MSI-H) via methylation of the DNA mismatch repair gene hMLH.3,4 It is generally believed that these cancers originate from serrated polyps, because this combination of alterations...
is frequent in serrated polyps (hyperplastic polyps, sessile serrated adenomas, mixed hyperplastic/adenomatous polyps, also known as admixed polyps, and traditional serrated adenomas), but absent in sporadic non-serrated adenomas. The serrated pathway has emerged as the second most significant pathway leading to CRC. A smaller subset of CRCs, at least 7.5%, can be distinguished by their morphology as being derived from serrated precursor lesions, even when such precursor lesions are no longer visible. We have referred to them as 'serrated adenocarcinoma' to indicate their origin in serrated polyps. Although many of these cancers retain a serrated pattern of epithelium, poorly differentiated ones are better characterized by abundant eosinophilic cytoplasm and a trabecular growth pattern.4,5

KRAS mutations have been considered to be the hallmark mutations of Vogelstein's adenoma–carcinoma model. Given the fact that the development of CRCs from serrated polyps with KRAS mutations has not yet been described, KRAS-mutated serrated polyps have been suggested to make a minor contribution to CRC development.6,7

KRAS and BAF belong to the intracellular RAS/RAF/MEK/mitogen-activated protein kinase (MAPK) cascade, which mediates cellular responses to growth signals. Activating mutations of KRAS occur in 30–50% of CRCs.8,9 Most (90%) are found in codons 12 and 13 of exon 1, and about 5% in codons 59 and 61 of exon 2.10 A single missense mutation of BRAF (BRAF V600E) accounts for 80% of the mutations in CRCs.11,12 Both BRAF and KRAS mutations have been found in the earliest detectable lesions with a serrated morphology, i.e. in hyperplastic/heteroplastic aberrant crypt foci.4,13 BRAF mutations have been reported in 19–36% of hyperplastic polyps, in 40–89% of admixed polyps, in 75–82% of sessile serrated adenomas, and in 20–66% of traditional serrated adenomas.3,4,14,15 Similarly, KRAS mutations have been reported in about 18% of aberrant crypt foci, in 4–37% of hyperplastic polyps, in 60% of admixed polyps, in up to 80% of traditional serrated adenomas, and in up to 10% of sessile serrated adenomas.4,16

A recent study based on 11 cases found BRAF mutations to be frequent and highly specific for serrated adenocarcinoma,17 but the significance of KRAS mutations in serrated adenocarcinoma development is not known. Therefore, this study was conducted in order to identify the prevalence of BRAF and KRAS mutations in serrated adenomas and serrated adenocarcinomas, and their potential associations with both the microsatellite instability (MSI) status and the methylation of hMLH1 and MGMT, which are known to be altered in serrated polyps and in the sporadic MSI/methylation pathway to CRC.

Materials and methods

Materials

Altogether, 47 serrated adenocarcinomas were obtained for this study. The histology was confirmed by two independent pathologists (M.J.M. and T.J.K.) from the haematoxylin and eosin-stained slides. Samples were diagnosed as serrated adenocarcinomas when the cancer tissue was composed of epithelial proliferation reminiscent of serrated adenoma, i.e. the cytoplasm of these cells was clear or eosinophilic, and when the cellular changes were accompanied by a serrated growth pattern, i.e. cells forming pseudopapillary or pseudocribriform structures with tufting of the cells into the lumen without true papillary structures with a fibrovascular core.5 CRC specimens with serrated morphology were screened from the previously described, consecutively collected and population-based Finnish collection of 466 samples.18 This material yielded 38 serrated cases, of which 35 were previously diagnosed as serrated cancers.5 After careful review, three cases were reclassified as serrated adenocarcinomas. These cases did not have a residual serrated adenoma component (see Figure 1B for an example). The original series of samples was extended to cover the years 1997–1998, raising the number of samples in the consecutive series to 552 and the number of serrated CRCs to 45. Two additional serrated cases removed between the years 2006 and 2007 were included. With five serrated cases of the consecutive series lacking sufficient sample material, 42/47 serrated CRCs were included in the DNA analyses of the present study. For the sake of simplicity, we have referred to all the other adenomas and carcinomas as 'non-serrated' in this paper.

A control series of non-serrated adenocarcinomas (n = 32) was selected from the demographic series of 552 cases to match gender, location, Dukes’ stage and World Health Organization histological grade. In the proximal colon, serrated adenocarcinomas represented such a large proportion that matched controls could not be obtained for all cases. Therefore, the matched control group remained smaller than the study group, and thus a set of 27 unmatched non-serrated adenocarcinomas selected from the same series to the study was added, yielding a total of 59 non-serrated adenocarcinomas.

Residual benign, serrated adenoma in contact with cancer tissue was present in 28/42 serrated
adenocarcinoma cases. For purposes of comparison, adenoma tissue was separately collected for the analyses. In nine cases of serrated adenocarcinoma, the residual serrated adenoma tissue was available for the analyses. An additional set of 17 serrated adenomas and nine non-serrated adenomas with a size of 5 mm or more was retrieved from the archives of Oulu University Hospital Department of Pathology.

DNA EXTRATION AND MSI ANALYSIS

DNA extraction and MSI analysis were performed as described previously. Tumour DNA was analysed with the NIH consensus marker panel, and distinction of MSI-H from microsatellite stability (MSS)/low-level MSI (MSI-L) was made according to the NIH consensus statement.
**Methylation Analysis**

Methylation analyses of the promoter sequences of the hMLH1 and MGMT genes were performed using the methylation-specific polymerase chain reaction (PCR) based on bisulphite pretreatment of DNA. The primer pairs used for the methylated and unmethylated templates of hMLH1 were TTTTTTAGGAGTGAAGGAGGT-TACG (forward) and GCCACTACGAAACTAAACACA- AA (reverse), and TTTTTAGGAGTGAAGGAGGTTATGG (forward) and AAACACCACTACAAAACTAAACACA-AA (reverse), respectively. The primer pairs used for the methylated and unmethylated templates of MGMT were TTTCGACGTTCGTAGGTTTTCG (forward) and GCAC- TCTTCCAAAACGAAACG (reverse), and TTTGTGT-TTGATGTTTGTAGGTTTTTGT (forward) and AACTCACACTCTTCCAAAACAAACA (reverse), respectively. Bisulphite-treated genomic DNA (100 ng) was used as a template in the PCR. The 2× JumpStart RED Taq ReadyMix (Sigma-Aldrich, St Louis, MO, USA) was used according to the manufacturer’s instructions. Commercial methylated DNA (CpGENOME Methylated DNA) and unmethylated DNA (CpGENOME Unmethylated DNA) were included in all the analyses as internal controls (Chemicon International, Temecula, CA, USA). The PCR products were visualized with UV illumination on a 2.5% agarose gel. The results of the methylation analyses of the hMLH1 and MGMT genes were compared with the intensity of the immunoreaction of the corresponding proteins.

**Mutation Analysis**

Mutation analyses of BRAF V600E and KRAS were performed by direct sequencing with PCR-amplified template DNA (25 ng), using a dynazyme DNA polymerase kit (Finnzymes Oy, Espoo, Finland). The primers used in the analysis were AAACCTTCATAATGTGCTTG-TCTG (forward) and GGCAAAATTTAATCAGTG-GA (reverse) for BRAF V600E, TGGTGGAGTATTT-GATAGTGTA (forward) and ATGGTCCTGACACCATATA (reverse) for KRAS c12/13, and TGAAGTAAAA-GTTGACTGTAATA (forward) and TAAACCCACC-TATAATGTTGAA (reverse) for KRAS c59/61. PCR conditions are available on request. The amplifications were performed with a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). The PCR products were enzymatically purified with EXOSAPit (USB, Cleveland, OH, USA) according to the manufacturer’s instructions. Sequencing of the product was performed in both directions on the ABI 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), with the forward and reverse primers. The data obtained were analysed with CHROMAS 1.6 sequencing analysis software (Technelysium Pty, Halensvale, Australia). All mutations were reconfirmed by independent PCR reactions and sequencing.

**Immunohistochemistry**

MLH1 and MSH2 analyses were carried out as described previously. Briefly, mouse monoclonal antibodies for MLH1 and MSH2 (BD-PharMingen, San Diego, CA, USA) were applied at a dilution of 1:25 (MLH1) or 1:50 (MSH2) for 1 h at room temperature. The reaction in the tumour area was considered to be negative if there was no staining in any of the tumour cell nuclei. For MGMT analysis, tissue sections were pretreated in 0.01 m citrate (pH 6.0) buffer in a microwave oven at 800 W for 2 min and at 300 W.

### Table 1. The success rates of DNA analyses

|                | Serrated CRCs | Matched non-serrated CRCs | All non-serrated CRCs | Serrated adenomas | Serrated adenomas adjacent to cancer | Non-serrated adenomas |
|----------------|---------------|---------------------------|-----------------------|------------------|-------------------------------------|-----------------------|
| BRAF V600E     | 42/42         | 31/32                     | 49/59                 | 17/17            | 9/9                                 | 9/9                   |
| K Ras c12/13   | 42/42         | 32/32                     | 45/59                 | 17/17            | 9/9                                 | 9/9                   |
| K Ras c59/61   | 40/42         | 31/32                     | 56/59                 | 17/17            | 8/9                                 | 9/9                   |
| MSI analyses   | 37/42         | 30/32                     | 56/59                 | 11/17            | 2/9                                 | 4/9                   |
| Methylation analyses hMLH1 | 27/42 | 29/32                     | 51/59                 | –                | –                                   | –                     |
| MGMT           | 29/42         | 29/32                     | 53/59                 | –                | –                                   | –                     |

CRC, Colorectal carcinoma.
for 10 min. Primary antibody for MGMT was applied at a dilution of 1:300 for 1 h at room temperature. A Dako EnVision kit (Dako, Copenhagen, Denmark) was used in the detection of the bound antibodies, with 3,3'-diaminobenzidine as a chromogen. The reaction in the tumour area was considered to be negative if less than 10% of the tumour cell nuclei stained positive.

**Statistical Analysis**

Tests were performed with statistical software (SPSS 16.1; SPSS, Chicago, IL, USA). The chi-square-test or Fisher’s exact test were used unless otherwise stated. A *P*-value of <0.05 was considered to be statistically significant.

**Table 2.** The clinical and pathological features of serrated and non-serrated adenocarcinomas

|                        | Serrated adenocarcinomas (n = 42) | Non-serrated adenocarcinomas (n = 59) | *P*-value (χ²-test) |
|------------------------|-----------------------------------|---------------------------------------|-------------------|
| **Mean age in years (range)** | 67.5 (43–85)                      | 68.5 (38–88)                          |                   |
| **Gender, N (%)**      |                                   |                                       |                   |
| Male                   | 17 (40.5)                         | 23 (39.0)                             | 0.880             |
| Female                 | 25 (59.5)                         | 36 (61.0)                             |                   |
| **Location, N (%)**    |                                   |                                       |                   |
| Proximal colon         | 24 (57.1)                         | 20 (33.9)                             | 0.050             |
| Distal colon           | 6 (14.3)                          | 9 (15.3)                              |                   |
| Rectum/rectosigmoid colon | 12 (28.6)                        | 30 (50.8)                             |                   |
| **Grade, N (%)**       |                                   |                                       |                   |
| I                      | 14 (33.3)                         | 11 (18.6)                             | 0.085             |
| II                     | 20 (47.6)                         | 41 (69.5)                             |                   |
| III                    | 8 (19.0)                          | 7 (11.9)                              |                   |
| **Dukes’ stage, N (%)**|                                   |                                       |                   |
| A                      | 6 (14.3)                          | 13 (22.0)                             | 0.699             |
| B                      | 20 (47.6)                         | 23 (39.0)                             |                   |
| C                      | 10 (23.8)                         | 16 (27.1)                             |                   |
| D                      | 6 (14.3)                          | 7 (11.9)                              |                   |
| **Mucinous, N (%)**    |                                   |                                       |                   |
| No                     | 26 (61.9)                         | 54 (91.5)                             | <0.001            |
| Yes                    | 16 (38.1)                         | 5 (8.5)                               |                   |
| **MSI**                |                                   |                                       |                   |
| MSI-H                  | 7 (18.9)                          | 4 (7.1)                               | 0.164             |
| MSS/MSI-L              | 30 (81.1)                         | 52 (92.9)                             |                   |

MSI, Microsatellite instability; MSI-H, high-level MSI; MSI-L, low-level MSI.

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BRAF mutations were frequent (33.3%; 14/42) and specific to serrated adenocarcinomas. After a careful histopathological re-evaluation of the cases, three BRAF-mutated cases, originally placed in the control group, were reclassified as serrated adenocarcinomas because the serrated morphology was preserved in the tumour. KRAS mutations were more frequent in serrated adenocarcinomas (45.2%) than in non-serrated adenocarcinomas (27.1%; P = 0.002). The higher frequency of KRAS mutations was clearly more evident (although not statistically significant) in cancers with a residual serrated adenoma (57.1%, 16/28), being observed twice as often as BRAF mutations (28.6%, 8/28). The combined prevalence of BRAF and KRAS mutations (78.6%) in serrated adenocarcinomas was higher than in non-serrated adenocarcinomas (27.1%; P < 0.001; Table 4), and the combined prevalence of BRAF and KRAS mutations in serrated adenocarcinomas with a residual serrated adenoma component reached 85.7% (24/28). In adenomas, BRAF mutations were specific to serrated adenomas, as none of the non-serrated adenomas showed a BRAF mutation (P = 0.058). Only one non-serrated adenoma carried a KRAS mutation. Either BRAF or KRAS mutation was observed in 82.4% of serrated adenomas (P < 0.001; Table 4).

Among non-serrated adenocarcinomas, KRAS c12/13 and c59 mutations were found in 16 cases (Table 4). The mutation pattern of KRAS c12/13 and c59/61 is shown in Table 5. The c12 G → A transitions were found in 52.6% (10/19) of KRAS-mutated serrated adenocarcinomas and in 12.5% (2/16; P = 0.047) of KRAS-mutated non-serrated cancers. This transition showed a distinct association with serrated adenocarcinomas, being present in 24% of cases (10/42), but in only 3.4% of non-serrated carcinomas (2/59; P = 0.001; Fisher’s exact test).

The MSI analyses in carcinoma material were successful in 93 cases (Table 1). Concurrent data from the MSI analyses and the KRAS/BRAF mutation analyses were obtained in 74 carcinoma cases (Table 6). Five BRAF mutations and one KRAS c61 mutation were observed among 11 MSI-H cancers when unmatched cases were included (P = 0.007) (Table 6). Serrated adenocarcinomas presenting with MSI-H were unlikely to be hereditary non-polyposis CRC cases. They occurred in old patients (Table 7) and presented with an adjacent sessile serrated adenoma (cases 1 and 5 in Table 7) or traditional serrated adenoma (cases 2, 3 and 6 in Table 7). Sixty-three MSS/MSI-L cancers showed an almost equal distribution of the wild-type cancers and KRAS-mutated cancers (P = 0.007; Table 6).

Seven of 34 serrated adenocarcinomas showed MSI-H (20.6%), and five of them (71.4%) had a concurrent BRAF mutation (P = 0.075; Table 6). One MSI-H case was wild type for both BRAF and KRAS, and another

|                | Serrated adenomas (n = 26)* | Non-serrated adenomas (n = 9) | P-value (χ²-test) |
|----------------|-------------------------------|-------------------------------|------------------|
| Mean age in years (range) | 64.1 (36–84)*                  | 74.1 (60–83)                  |                   |
| Gender, N (%) |                                |                               |                  |
| Male           | 13 (50.0)                     | 8 (90)                        | 0.040            |
| Female         | 13 (50.0)                     | 1 (10)                        |                  |
| Location, N (%)|                              |                               |                  |
| Proximal colon | 6 (20)                        | 0 (0)                         | 0.396            |
| Distal colon   | 4 (5)                         | 2 (20)                        |                  |
| Rectum/rectosigmoid colon | 16 (75)                 | 7 (80)                        |                  |
| Dysplasia, N (%)|                              |                               |                  |
| Mild           | 6 (35)                        | 3 (40)                        | 0.024            |
| Moderate       | 8 (25)                        | 6 (60)                        |                  |
| Severe         | 12 (40)                       | 0 (0)                         |                  |

*All serrated adenomas, including adenomas adjacent to cancer (n = 9).
showed a KRAS mutation at codon 61. KRAS c12/13 mutations in serrated adenocarcinomas were never accompanied by MSI-H, in contrast to 15/27 (55.6%) of MSS/MSI-L cases harbouring a KRAS mutation (P = 0.075). In non-serrated adenocarcinomas, MSI-H and KRAS mutations did not co-occur (P = 0.278). Analyses with the matched controls yielded similar results (not shown).

Promoter methylation analysis was successful in 78/101 cases for hMLH1 and in 82/101 cases for MGMT (Table 1). The relationships between BRAF and KRAS mutations and hMLH1 and MGMT methylation...
status are summarized in Table 8. BRAF mutations were tightly associated with hMLH1 and MGMT methylation, whereas KRAS mutations had a negative correlation with hMLH1 and MGMT methylation (Table 8). The loss of MGMT expression in immunohistochemistry was associated with the corresponding methylation of the MGMT gene ($P < 0.0001$; Table 9). In MSS cancers, the presence of hMLH1 and MGMT methylation did not correspond to the loss of immunohistochemical expression (not shown), suggesting incomplete methylation of hMLH1.

Our findings fit relatively well with the molecular classification of CRC proposed by Jass 6 (Table 10). We were able to show that Jass groups 1 and 2 definitely represent serrated adenocarcinomas, but KRAS-mutated cases belonging to Jass group 3 were composed of serrated and non-serrated cancers. Most Jass group 4 tumours could be classified as non-serrated, and most cases in Jass group 5 were probably Lynch syndrome cases, except for one case presenting with a typical serrated growth pattern and residual serrated adenoma (illustrated in Figure 1G,H).

**Discussion**

This is the first study to show that KRAS mutations are frequent (45%) in serrated adenocarcinomas, and that the MAPK activation resulting from either KRAS or BRAF mutations is very common (79%) in serrated adenocarcinomas. Earlier studies have claimed that BRAF predominates over KRAS in biological significance in the serrated pathway, 17,23 because: (i) BRAF mutations are specific to serrated polyps and serrated adenocarcinoma; (ii) malignant serrated endpoints presenting with KRAS mutations have not been reported until now; and (iii) there has been no previous evidence that KRAS-mutated CRCs emerge from two separate molecular pathways.

Sporadic MSI-H cancers have been attributed to the serrated pathway. 5,14,17,24,25 In our study, MSI-H was seen in only 20.6% of serrated adenocarcinomas. A distinct association of BRAF mutations with MSI-H and the methylation of hMLH1 and MGMT was observed among serrated cases, thus corroborating the idea that BRAF-mutated CRCs (Jass groups 1 and 2) represent serrated adenocarcinomas with high accuracy, 6,14,26 but the relatively low frequency of MSI-H cancers among serrated adenocarcinomas indicates that sporadic MSI-H colorectal cancers can be attributed only to a subset of serrated adenocarcinomas. 4,5,14

The serrated adenocarcinoma cases presenting with a residual adenoma component undoubtedly showed KRAS mutations (57.1%) to be twice as frequent as
BRAF mutations (28.6%) in serrated adenocarcinomas. Either KRAS or BRAF mutation was observed in 85.7% of these cases, and in 82.4% of the serrated adenomas. These numbers suggest that MAPK activation is central for the serrated adenocarcinoma pathway, and that many CRCs with KRAS mutations, MSS/MSI-L and less frequent DNA hypermethylation originate from serrated polyps.

Table 6. The prevalences of BRAF V600E and KRAS mutations in serrated and non-serrated adenomas and adenocarcinomas with high-level microsatellite instability (MSI-H) and microsatellite stability (MSS)/low-level microsatellite instability (MSI-L)

| Mutation                        | Mutations   | All, N | MSS/MSI-L, n (%) | MSI-H, n (%) | P-value (Fisher’s exact test) |
|---------------------------------|-------------|--------|------------------|--------------|-------------------------------|
| **All CRC**                     |             |        |                  |              |                               |
| BRAF V600E mutation             | 12          | 7 (58.3) | 5 (41.7)         | 0.007        |
| KRAS c12/13 or c59/61 mutation  | 31          | 30 (96.8) | 1 (3.2)          |              |
| Wild type                       | 31          | 26 (83.9) | 5 (16.1)         |              |
| **Serrated CRC with their matched controls** | | | | | |
| BRAF V600E mutation             | 12          | 7 (58.3) | 5 (41.7)         | 0.008        |
| KRAS c12/13 or c59/61 mutation  | 29          | 28 (96.5) | 1 (3.5)          |              |
| Wild type                       | 23          | 18 (78.3) | 5 (21.7)         |              |
| **Serrated CRC**                |             |        |                  |              |                               |
| BRAF V600E mutation             | 12          | 7 (58.3) | 5 (41.7)         | 0.075        |
| KRAS c12/13 or c59/61 mutation  | 16          | 15 (93.8) | 1 (6.2)          |              |
| Wild type                       | 6           | 5 (83.3)  | 1 (16.7)         |              |
| **Non-serrated CRC**           |             |        |                  |              |                               |
| BRAF V600E mutation             | 0           | 0 (0)   | 0 (0)            | 0.278        |
| KRAS c12/13 or c59/61 mutation  | 15          | 15 (100) | 0 (0)            |              |
| Wild type                       | 25          | 21 (84)  | 4 (16.0)         |              |
| **Matched non-serrated CRCs**    |             |        |                  |              |                               |
| BRAF V600E mutation             | 0           | 0 (0)   | 0 (0)            | 0.113        |
| KRAS c12/13 or c59/61 mutation  | 13          | 13 (100) | 0 (0)            |              |
| Wild type                       | 17          | 13 (76.5) | 4 (23.5)         |              |
| **All adenomas**                |             |        |                  |              |                               |
| BRAF V600E mutation             | 4           | 4 (100)  | 0 (0)            | 0.588        |
| KRAS c12/13 or c59/61 mutation  | 7           | 7 (100)  | 0 (0)            |              |
| Wild type                       | 6           | 5 (83.3)  | 1 (16.7)         |              |
| **Serrated adenomas**           |             |        |                  |              |                               |
| BRAF V600E mutation             | 4           | 4 (100)  | 0 (0)            | NA           |
| KRAS c12/13 or c59/61 mutation  | 7           | 7 (100)  | 0 (0)            |              |
| Wild type                       | 2           | 2 (100)  | 0 (0)            |              |
| **Non-serrated adenomas**       |             |        |                  |              |                               |
| BRAF V600E mutation             | 0           | 0 (0)   | 0 (0)            | NA           |
| KRAS c12/13 or c59/61 mutation  | 0           | 0 (0)   | 0 (0)            |              |
| Wild type                       | 4           | 3 (75)   | 1 (25.0)         |              |

CRC, Colorectal carcinoma; NA, not applicable.

KRAS mutations have generally been considered to be characteristic of Vogelstein’s adenoma–carcinoma model, and the integration of KRAS mutation in the model was justified by the high frequency of KRAS mutations in CRCs. Recent, well-conducted studies based on extensive case series – carried out after the recognition of serrated adenomas – have repeatedly found that KRAS mutations are rare in tubular
Table 7. Features of serrated (n = 7) and non-serrated (n = 4) cancers with high-level microsatellite instability with respect to cancer and family history, mutation status of *KRAS/BRAF*, *MLH1/MSH2* immunohistochemistry, and h*MLH1* methylation

| Case | Type of carcinoma | Age (years) | Family history of cancer | Other cancers in patient | Mutation status of *KRAS/BRAF* | hMLH1 immunohistochemistry | MSH2 immunohistochemistry | hMLH1 methylation |
|------|-------------------|-------------|--------------------------|--------------------------|-------------------------------|---------------------------|---------------------------|-----------------|
| 1    | Serrated          | 78          | Not known                | No                       | Wild type                     | –                         | –                         | No              |
| 2    | Serrated          | 85          | Not known                | No                       | *BRAF* V600E                  | –                         | +                         | Yes             |
| 3    | Serrated          | 84          | Not known                | Yes, skin                | *KRAS* c59/61                 | –                         | +                         | Yes             |
| 4    | Serrated          | 71          | Not known                | Yes, breast              | *BRAF* V600E                  | –                         | +                         | Yes             |
| 5    | Serrated          | 68          | Yes, CRC                 | No                       | *BRAF* V600E                  | –                         | +                         | Yes             |
| 6    | Serrated          | 60          | Not known                | No                       | *BRAF* V600E                  | +                         | +                         | Yes             |
| 7    | Serrated          | 83          | Not known                | No                       | *BRAF* V600E                  | –                         | +                         | Yes             |
| 8    | Non-serrated      | 73          | Not known                | No                       | Wild type                     | –                         | +                         | No              |
| 9    | Non-serrated      | 72          | Not known                | No                       | Wild type                     | –                         | +                         | No              |
| 10   | Non-serrated      | 71          | Not known                | No                       | Wild type                     | +                         | –                         | No              |
| 11   | Non-serrated      | 53          | Not known                | Yes, breast              | Wild type                     | –                         | +                         | No              |

CRC, Colorectal carcinoma.
adenomas, which constitute 85–90% of colorectal non-serrated adenomas.\textsuperscript{27,28} Barry \textit{et al.}\textsuperscript{28} documented a 3% frequency of KRAS mutations in a prospective study of 303 adenomas, most mutations being observed in sessile and tubulovillous adenomas. Only two of 259 tubular adenomas (0.8%) harboured a KRAS mutation. Maltzman \textit{et al.}\textsuperscript{27} reported a 10.6% frequency for KRAS mutations in tubular adenomas.

The high frequency of KRAS mutations in serrated adenomas and serrated adenocarcinomas observed in the present study explains, in part, why KRAS mutations are less frequent in non-serrated adenomas but occur in about 40% of CRCs.

The high frequency of KRAS mutations in serrated adenocarcinomas further strengthens the importance of the colorectal serrated pathway. The estimated 15–20% frequency of serrated adenocarcinomas is based on the frequency of sporadic BRAF-mutated, CIM-positive CRCs.\textsuperscript{8} If KRAS-mutated serrated adenocarcinomas were taken into account, the proportion of the serrated pathway could reach 30% of all CRCs: KRAS mutations are more frequent in serrated polyps than in non-serrated adenomas, being observed in up to 37% of hyperplastic polyps, in up to 60% of admixed polyps, and in up to 80% of traditional serrated adenomas.\textsuperscript{4,16}

The given frequencies of 30–50% for KRAS mutations for all colorectal cancers and 45% for serrated adenocarcinomas allow an assumption that 15–30% of KRAS-mutated CRCs may evolve from serrated adenomas, if we consider that the serrated pathway represents 15–20% of colorectal cancers.\textsuperscript{4} A similar conclusion can be drawn on the basis of polyp demographics. If traditional serrated adenomas represent 3% and non-serrated adenomas 85% of all polyps, then the 80% KRAS mutation rate in traditional serrated adenomas and the 3% mutation rate in non-serrated adenomas would yield KRAS-mutated polyps in roughly equal numbers (2.4% and 2.6% of all polyps, respectively).

It must be emphasized that serrated adenocarcinoma has not been considered as an entity in any of the
Table 9. The correlation of MGMT/hMLH1 promoter methylation with the immunoreaction of the corresponding proteins in all cancers, serrated cancers and their matched controls

| Promoter methylation of MGMT/hMLH1 | MGMT expression* | hMLH1 expression | P-value (Fisher’s exact test) |
|-----------------------------------|-----------------|-----------------|-----------------------------|
|                                   | All, N | Positive | Negative | All, N | Positive | Negative | P-value (Fisher’s exact test) |
| All cases                        |       |          |          |       |          |          |                              |
| Unmethylated                     | 45    | 45       | 0        | 52    | 48       | 4        | <0.0001                      |
| Unmethylated and methylated      | 27    | 21       | 6        | 25    | 21       | 4        | 0.086                         |
| Methylated                       | 11    | 5        | 6        | 5     | 3        | 2        | 0.086                         |
| Serrated CRC                    |       |          |          |       |          |          |                              |
| Unmethylated                     | 13    | 13       | 0        | 13    | 12       | 1        | 0.041                         |
| Unmethylated and methylated      | 17    | 13       | 4        | 14    | 11       | 3        | 0.186                         |
| Methylated                       | 4     | 2        | 2        | 4     | 2        | 2        | 0.186                         |
| Matched controls                |       |          |          |       |          |          |                              |
| Unmethylated                     | 16    | 16       | 0        | 24    | 21       | 3        | 0.013                         |
| Unmethylated and methylated      | 6     | 4        | 2        | 4     | 3        | 1        | 0.553                         |
| Methylated                       | 6     | 3        | 3        | 1     | 1        | 0        | 0.553                         |

For *MGMT immunoreaction, tumour tissue presenting over 10% of positive cells was considered to be positive.

CRC, Colorectal carcinoma.
previous studies on the frequency and pathogenesis of KRAS-mutated CRCs.\textsuperscript{29–31} This should be kept in mind when interpreting previous data on the KRAS mutation rate in CRCs. The Vogelstein adenoma–carcinoma model, published in 1990, was constructed ahead of the description of serrated adenomas in the same year. Therefore, it is likely that the Vogelstein model was originally contaminated by observations on (traditional) serrated adenomas bearing KRAS mutations misclassified as non-serrated adenomas.

The most frequent KRAS mutation in codons 12/13 are c12 2G → A (31–38%), c12 2G → T (21–31%), c13 2G → A (13–21%), and c12 1G → T (7–10%).\textsuperscript{29–31} The relative proportions of specific types of KRAS mutations that we identified were similar to those previously described (Table 5). However, the c12 G → A transition was almost completely specific to serrated adenocarcinomas, being present in 24% (10/42) of the cases, whereas in non-serrated carcinomas it was present in only 3% (2/59) of cases (P = 0.001). Therefore, it is likely that many CRCs with the KRAS c12 G → A transversion represent serrated adenocarcinomas. Besides providing a potential genetic marker for the serrated adenocarcinomas, the specificity of the c12 G → A transition may indicate the occurrence of specific aetiological factors, such as endogenous environmental or endogenous alkylating agents.\textsuperscript{32,33} The specificity of G → A transitions to serrated adenocarcinomas justifies the analysis of specific environmental risk factors, such as smoking, as possible causative agents.

In conclusion, the cumulative 79–82% frequency of BRAF and KRAS mutations in serrated adenocarcinoma and its precursors observed in our study underlines the importance of MAPK pathway activation in the serrated pathway, and suggests that these mutations are the driver mutations in the serrated pathway. The co-occurrence of BRAF mutations, CIM and MSI-H represents an easily identifiable subset of serrated adenocarcinoma, and BRAF mutation analysis can be utilized to detect these cases. However, the high frequency of KRAS mutations, particularly the c12 G → A transition, in serrated neoplasms emphasizes that KRAS mutation is an even more important alteration in the serrated pathway. Many KRAS-mutated CRCs originate from serrated polyps, and complete removal and follow-up of serrated adenomas with KRAS mutations is therefore essential to reduce the total CRC burden. The high frequency of KRAS mutations in serrated adenocarcinomas also indicates that most serrated adenocarcinomas are natively insensitive to epidermal growth factor receptor-blocking therapies, which are increasingly being used to treat metastatic colorectal cancer.\textsuperscript{1,14}

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Conflict of interest

There are no conflicts of interest.

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