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

Short title: *KRAS* and *BRAF* in serrated adenocarcinoma

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ABSTRACT

Aims: To define the occurrence of *KRAS* and *BRAF* mutations, microsatellite instability (MSI), *MGMT* and *hMLH1* methylation and expression in colorectal serrated adenocarcinoma. **Methods:** *KRAS* codon 12/13 and 59/61 and *BRAF*V600E mutations, MSI, *MGMT* and *hMLH1* methylation and expression in 42 serrated adenocarcinomas and 17 serrated adenomas was compared with 59 non-serrated CRCs and 9 adenomas. **Results:** *KRAS* and *BRAF* mutations were observed in 45% and 33% of serrated adenocarcinomas and in 27% and 0% of non-serrated CRCs ($p<0.001$). *KRAS* c12G→A transition was the predominant type of mutation in serrated adenocarcinomas. *BRAF*-mutated serrated adenocarcinomas were MSI-H in 42% ($p=0.075$), showed *hMLH1* (100%; $p=0.001$) and *MGMT* methylation (90.9%; $p=0.019$). Fifty-six percent of MSS/MSI-L serrated adenocarcinomas harboured *KRAS* mutations. In non-serrated cancers, *KRAS* mutations did not associate with MSI status. **Conclusions:** High combined mutation rate (79-82%) of *KRAS* and *BRAF* in serrated adenomas and adenocarcinomas indicates that MAPK activation is a crucial part of 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. 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 Vogelstein's model.

Key Words: colorectal cancer, serrated adenocarcinoma, DNA hypermethylation, microsatellite instability, *KRAS*, *BRAF*, *MGMT*, *hMLH1*

INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer type in the Western world¹. For a long time, non-serrated adenomas were thought to represent the only significant precursor lesion for CRC². However, it is now apparent that the development of 15-20% of sporadic CRCs is not explained by the 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 the 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 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 characterised with an abundant eosinophilic cytoplasm and a trabecular growth pattern.^{4,5}

KRAS mutations have been considered to be the hallmark mutations of the Vogelstein's adenoma-carcinoma model. Adding this to the fact that the development of CRC from serrated polyps with *KRAS* mutations has not yet been described, *KRAS* mutated serrated polyps have been suggested to have a minor contribution to CRC development^{6,7}.

KRAS and *BRAF* belong to the intracellular RAS/RAF/MEK/MAP kinase (MAPK) cascade that 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 2¹⁰. A single missense mutation of *BRAF* (*BRAF*V600E) accounts for 80% of the mutations in CRC^{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 (ACF-H)^{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 eleven cases documented *BRAF* mutations to be frequent and highly specific for serrated adenocarcinoma¹⁷, but the significance of *KRAS* mutations in serrated adenocarcinoma development is not known. Therefore, we conducted this study in order to identify the prevalence of *BRAF* and *KRAS* mutations in serrated adenomas and serrated adenocarcinomas and their potential associations with both the MSI status and the methylation of *hMLH1* and *MGMT*, known to be altered in serrated polyps and in the sporadic MSI/methylator pathway to CRC.

MATERIALS AND METHODS

Materials. Altogether, 47 serrated adenocarcinomas were obtained for this study. The histology was confirmed by two independent pathologists (MJM, TJK) 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 with 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 core⁵. CRC specimens with serrated morphology were screened from the previously described, consecutively collected and population-based Finnish collection of 466 samples¹⁸. This material yielded 38 serrated cases, of which 35 were previously diagnosed as serrated cancers⁵. Three cases were reclassified after careful review as serrated adenocarcinomas. These cases did not have a residual serrated adenoma component (See fig.1B for an example). The original series of samples was extended to cover the years 1997-98, raising the amount of samples in the consecutive series to 552 and the amount of serrated CRCs to 45. Two additional serrated cases removed between the years 2006-2007 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 all the other adenomas and carcinomas as "non-serrated" in this study.

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 WHO 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 we added a set of 27 unmatched non-serrated adenocarcinomas selected from the same series to the study, yielding a total of 59 non-serrated adenocarcinomas.

A benign, serrated adenoma residual 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 extraction and MSI analysis. DNA extraction and MSI analysis were performed as described earlier^{5,19}. Tumour DNA was analysed with the NIH consensus marker panel, and distinction of MSI-H from MSS/MSI-L was made according to the NIH consensus statement²⁰.

Methylation analysis. Methylation analyses of the promoter sequences of the *hMLH1* and *MGMT* genes were performed using the methylation-specific PCR²¹ based on bisulphite pretreatment of DNA. The primer pairs used for the methylated and unmethylated templates of *hMLH1* were TTTTGTAGGAGTGAAGGAGGTTACG (forward) and GCCACTACGAAACTAAACACGAA (reverse) and TTTTGTAGGAGTGAAGGAGGTTATGG (forward) and AAACACCACTACAAAACAAACACAAA (reverse), respectively. The primer pairs used for the methylated and unmethylated templates of *MGMT* were TTTCGACGTTTCGTAGGTTTTCGC (forward) and GCACTCTTCCGAAAACGAAACG (reverse)³² and TTTGTGTTTTGATGTTTGTAGGTTTTTGT (forward) and AACTCCACACTCTTCCAAAACAAAACA (reverse), respectively. Bisulphite-treated genomic DNA (100 ng) was used as a template in the PCR.

2x JumpStart REDTaq ReadyMix (Sigma-Aldrich Co., St. Louis, Missouri) was used according to the manufacturer's instructions. Commercial methylated (CpGENOME Methylated DNA) and unmethylated (CpGENOME Unmethylated DNA) DNA were included in all the analyses as internal controls (Chemicon International, Inc., Temecula, California). The PCR products were visualised with UV illumination on 2.5% agarose gel. The results of the methylation analyses of the *hMLH1/MGMT* genes were compared with the intensity of the immunoreaction of the corresponding proteins⁵.

Mutation analysis. Mutation analyses of *BRAFV600E* 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 AAACCTCTTCATAATGCTTGCTCTG (forward) and GGCCAAAAATTTAATCAGTGGA (reverse) for *BRAFV600E*, TGGTGGAGTATTTGATAGTGTA (forward) and ATGGTCCTGCACCAGTAATA (reverse) for *KRAS*_{12/13} and TGAAGTAAAAGGTGCACTGTAATA (forward) and TAAACCCACCTATAATGGTGAA (reverse) for *KRAS*_{59/61}. PCR conditions are available on request. The amplifications were performed with a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts). The PCR products were enzymatically purified with EXOSAPit® (USB, Cleveland, Ohio) according to the manufacturer's instructions. Sequencing of the product was performed in both directions on the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California) using the forward and reverse primers. The data obtained were analysed with the Chromas 1.6 sequencing analysis software (Technelysium Pty Ltd, Halensvale, Australia). All mutations were reconfirmed by independent PCR reactions and sequencing.

Immunohistochemistry. MLH1 and MSH2 analyses were carried out as described earlier⁵. Briefly, mouse monoclonal antibodies for MLH1 and MSH2 (BD-PharMingen, San Diego, CA) were applied at a dilution of 1:25 (MLH1) or 1:50 (MSH2), for one hour at room temperature. The reaction in the tumour area was considered 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 two minutes and at 300 W for ten minutes. Primary antibody for MGMT was applied at a dilution of 1:300 for one hour at room temperature. Dako EnVision kit (Dako, Copenhagen, Denmark) was used in the detection of the bound antibodies using 3,3'-diaminobenzidine as a chromogen. The reaction in the tumour area was considered negative, if less than 10% of the tumour cell nuclei stained positive.

Statistical analysis. Tests were performed using a statistical software (SPSS16.1; SPSS Inc., Chicago, Illinois). χ^2 test or Fisher's exact test were used unless otherwise stated. A p-value of less than 0.05 was considered statistically significant.

Ethical aspects. The protocol (58/2002) was approved by the Ethical Committee of Oulu University Hospital.

RESULTS

The success rate of each DNA analysis is shown in Table 1. Clinicopathological features of the study material are presented in Table 2, and examples of serrated adenocarcinomas in relation to *KRAS* and *BRAF* mutations and MSI status are illustrated in Figure 1. *BRAF* and *KRAS* mutations did not co-exist. The prevalence and distribution patterns of *BRAF*V600E and *KRAS*Sc12/13 and

c59/61 mutations are presented in Table 3. *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 classified to 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 cancers (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 ones (27.1%; $p<0.001$; Table 3), while the combined prevalence of *BRAF* and *KRAS* mutations in serrated adenocarcinomas with a residual serrated adenoma component was up to 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 3).

In non-serrated adenocarcinomas, *KRAS* codon 12/13 and 59 mutations were found in 16 cases (Table 3). The mutation pattern of *KRAS* c12/13 and c59/61 is shown in Table 4. 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 5). Five *BRAF* mutations and one *KRAS* c61 mutation were observed among eleven MSI-H cancers when also unmatched cases were included ($p=0.007$) (Table 5). Serrated adenocarcinomas presenting with MSI-H were unlikely HNPCC cases. They occurred in old patients (Table 6) and presented with an adjacent sessile serrated adenoma (cases 1 and 5 in Table 6) or traditional serrated adenoma (cases 2,3,6 in Table 6). Sixty-three MSS/MSI-L cancers showed an almost equal distribution of the wild type cancers and *KRAS* mutated cancers ($p=0.007$, Table 5).

Seven out of 34 serrated adenocarcinomas showed MSI-H (20.6%), and five of them (71.4%) had a concurrent *BRAF* mutation ($p=0.075$; Table 5). One MSI-H case was wild type for both *BRAF* and *KRAS*, and another showed a *KRAS* mutation at codon 61. *KRAS* c12/13 mutations in serrated adenocarcinomas were never accompanied with 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 for *hMLH1* was successful in 78/101 cases and for *MGMT* in 82/101 cases (Table 1). The relationships between *BRAF* and *KRAS* mutations and *hMLH1* and *MGMT* methylation status are summarised in Table 7. *BRAF* mutations were tightly associated with *hMLH1* and *MGMT* methylation, whereas *KRAS* mutations had a negative correlation to *hMLH1* and *MGMT* methylation (Table 7). The loss of *MGMT* expression in immunohistochemistry was associated with the corresponding methylation of the *MGMT* gene ($p<0.0001$, Table 8). 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 to the molecular classification of CRC proposed by Jass ⁶ (Table 9). 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 fitting to Jass group 5 were likely Lynch syndrome cases, except one case presenting with a typical serrated growth pattern and residual serrated adenoma (illustrated in Figure 1 G&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^{17,22}, because a) *BRAF* mutations are specific to serrated polyps and serrated adenocarcinoma, and b) malignant serrated endpoints presenting with *KRAS* mutations have not been reported until now, and c) there has not been previous evidence that *KRAS*-mutated CRC emerge from two separate molecular pathways.

Sporadic MSI-H cancers have been attributed to the serrated pathway^{5,14,17,23,24}. In our study, MSI-H was seen only in 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 CRC (Jass groups 1 and 2) represent serrated

adenocarcinomas with a high accuracy^{14,25,6}, 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 two times more frequent than *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 the 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.

KRAS mutations have generally been considered a 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 CRC. 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 adenomas, which constitute 85-90% of colorectal non-serrated adenomas^{26,27}. Barry *et al.* 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 out of 259 tubular adenomas (0.8%) harboured a *KRAS* mutation²⁷. Maltzman *et al.* reported a 10.6% frequency for *KRAS* mutations in tubular adenomas²⁶. The high frequency of *KRAS* mutations in serrated adenomas and serrated adenocarcinomas observed in the present study in part explains why *KRAS* mutations are less frequent in non-serrated adenomas but occur in around 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, CIMP-positive CRCs⁴. When *KRAS*-mutated serrated adenocarcinomas are 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^{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 serrated pathway represents 15-20% of colorectal cancer⁴. A similar conclusion can be made 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 emphasised that serrated adenocarcinoma has not been considered as an entity in any of the previous studies on the frequency and pathogenesis of *KRAS*-mutated CRCs²⁸⁻³⁰. This should be kept in mind when interpreting previous data on *KRAS*-mutation rate in CRCs. The Vogelstein adenoma-carcinoma model published in 1990 was constructed ahead of the description of serrated adenomas 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 5G→A (13-21%) and c12 1G→T (7-10%)²⁸⁻³⁰. The relative proportions of specific types of *KRAS* mutations we identified were similar to those previously described (Table 4). However, the c12 G→A transition was almost specific to serrated adenocarcinomas, being present in 24% (10/42) of the cases, whereas in non-serrated carcinomas it was present only in 3% (2/59) of cases (p=0.001). Therefore it is likely that many CRCs with *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 occurrence of specific aetiological factors, such as endogenous environmental or endogenous alkylating agents^{31,32}. 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. Co-occurrence of *BRAF* mutations, CIM and MSI-H represents an easily identifiable subset of serrated adenocarcinoma, and *BRAF* mutation analysis can be utilised to detect these cases. However, the high frequency of *KRAS* mutations, particularly the c12 G→A transition, in serrated neoplasms emphasises that *KRAS* mutation is an even more important alteration in the serrated pathway. Many *KRAS*-mutated CRCs originate from serrated polyps and therefore complete removal and follow-up of serrated adenomas with *KRAS* mutations is essential to reduce the risk of total CRC burden. High frequency of *KRAS* mutations in serrated adenocarcinomas also indicates that most serrated adenocarcinomas are natively insensitive to EGF receptor-blocking therapies, which are increasingly used to treat metastatic colorectal cancer^{33,34}.

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TABLES

Table 1. The success rates of DNA analyses.

		Serrated CRC	Matched non-serrated CRC	All non-serrated CRC	Serrated Adenomas	Serrated adenomas adjacent to cancer	Non-serrated adenomas
<i>BRAF</i> V600E		42/42	31/32	49/59	17/17	9/9	9/9
<i>KRAS</i> c12/13		42/42	32/32	45/59	17/17	9/9	9/9
<i>KRAS</i> 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	<i>hMLH1</i>	27/42	29/32	51/59	-	-	-
	<i>MGMT</i>	29/42	29/32	53/59	-	-	-

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

	<i>Serrated carcinomas (n=42)</i>		<i>Non-serrated carcinomas (n=59)</i>		<i>p value (χ^2 test)</i>
	Mean	Range	Mean	Range	
Age in years	67.5	43-85	68.5	38-88	
	N	%	N	%	
Gender					
Male	17	40.5	23	39.0	0.880
Female	25	59.5	36	61.0	
Location					
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					
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					
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					
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	
	<i>Serrated adenomas (n = 26)*</i>		<i>Non-serrated adenomas (n=9)</i>		<i>p value (χ^2 test)</i>
	Mean	Range	Mean	Range	
Age years (range)	64.1*	36-84	74.1	60-83	
	N	%	N	%	
Gender					
Male	13	50.0	8	90	0.040
Female	13	50.0	1	10	
Location					
Proximal colon	6	20	0	0	0.396
Distal colon	4	5	2	20	
Rectum/rectosigmoid colon	16	75	7	80	
Dysplasia					
Mild	6	35	3	40	0.024
Moderate	8	25	6	60	
Severe	12	40	0	0	

Explanations: * = all serrated adenomas, including adenomas adjacent to cancer (n=9)

Table 3. The prevalence and distribution of *BRAF*V600E and *KRAS* (codons 12/13 and 59/61)

			<i>BRAF</i> V600E	<i>KRAS</i> (all)	<i>KRAS</i> Sc12/13	<i>KRAS</i> Sc59/61	<i>Either BRAF or KRAS</i>
<i>Serrated adenocarcinomas (n=42)</i>	No adjacent adenoma component (n=14)	with mutation /all	6/14	3/14	2/14	1/14	9/14
		%	42.9	21.4	14.3	7.1	64.3
	Adjacent serrated adenoma (n=28)	with mutation /all	8/28	16/28	15/28	1/28	24/28
		%	28.6	57.1	53.6	3.6	85.7
	All (n=42)	with mutation /all	14/42	19/42	17/42	2/42	33/42
		%	33.3	45.2	40.5	4.8	78.6
<i>Matched non-serrated carcinomas (n=32)</i>		with mutation /all	0/32	13/32	12/32	1/32	13/32
		%	0	40.6	37.5	3.1	40.6
		<i>p</i>	<0.001	0.894	1.00	0.842	<0.001
<i>All non-serrated carcinomas (n=59)</i>		with mutation /all	0/59	16/59	15/59	1/59	16/59
		%	0	27.1	25.4	1.7	27.1
		<i>p</i>	<0.001	0.002	0.002	0.848	<0.001
<i>Serrated adenomas (n=17)</i>		with mutation /all	7/17	7/17	6/17	1/17	14/17
		%	41.2	41.2	35.3	5.9	82.4
<i>Non-serrated adenomas (n=9)</i>		with mutation /all	0/9	1/9	1/9	0/9	1/9
		%	0	11.1	11.1	0	11.1
		<i>p</i>	0.058	0.243	0.453	1	<0.001
<i>Serrated adenomas adjacent to cancer (n=9)</i>		with mutation /all	2/9	4/9	3/9	1/9	6/9
		%	22.2	44.4	33.3	11.1	66.7
		<i>p</i>	0.068	0.353	0.6	0.436	0.001

mutations according to the type of the neoplasm.

Table 4. The observed sequence changes and the corresponding amino acid substitutions in *KRAS* c12/13 and c59/61 in the study population.

<i>KRAS c12/13 and c59/61</i>	<i>Amino acid</i>	<i>Serrated</i>	<i>Non-</i>	<i>Total</i>	<i>p value</i>	<i>Serrated</i>	<i>Non-serrated</i>	<i>Total</i>	<i>p value</i>
<i>sequence change</i>	<i>change</i>	<i>adenoca.</i>	<i>serrated</i>		(Fisher's	<i>adenomas</i>	<i>adenomas</i>		(Fisher's
			<i>adenoca.</i>		exact test)				exact test)
c12 GGT→GTT	Gly→Val	4 (21.1%)	7(43.8%)	11(31.4%)		4 (36.4%)	0 (0)	4 (33.3%)	
c12 GGT→TGT	Gly→Cys	1(5.3%)	3(18.8%)	4(11.4%)		0 (0)	0 (0)	0 (0)	
c12 GGT→GAT	Gly→Asp	10(52.6%)	2(12.5%)	12(34.3%)		4 (36.4%)	1 (100%)	5 (41.7%)	
c13 GGC→GAC	Gly→Asp	2(10.5%)	3(18.8%)	5(14.3%)	0.047	1 (9.1%)	0 (0)	1 (8.3%)	1.00
c59 GCA→GGA	Ala→Gly	0(0)	1(6.3%)	1(2.9%)		0 (0)	0 (0)	0 (0)	
c61 CAA→AAA	Gln→Lys	2(10.5%)	0(0)	2(5.7%)		2 (18.2%)	0 (0)	2 (16.7%)	
Total		19(100%)	16(100%)	35(100%)		11(100%)	1(100%)	12(100%)	

Table 5. The prevalences of *BRAF*V600E and *KRAS* mutations in MSI-H and MSS/MSI-L serrated and non-serrated adenomas and adenocarcinomas.

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

Explanations: NA= Not applicable.

Table 6. Features of serrated (n=7) and non-serrated (n=4) MSI-H cancers in respect to cancer and family history, mutation status of *KRAS/BRAF* and *MLH1/MSH2* immunohistochemistry and *hMLH1* methylation.

Case	Type of carcinoma	Age	Family history of cancer	Other cancers in patient	Mutation status <i>KRAS/BRAF</i>	<i>hMLH1</i> immunohistochemistry	<i>MSH2</i> immunohistochemistry	<i>hMLH1</i> methylation
1	Serrated	78	Not known	No	Wt		-	No
2	Serrated	85	Not known	No	<i>BRAFV600E</i>	-	+	Yes
3	Serrated	84	Not known	Yes, skin	<i>KRAS</i> c59/61	-	+	Yes
4	Serrated	71	Not known	Yes, Breast	<i>BRAFV600E</i>	-	+	Yes
5	Serrated	68	Yes, CRC	No	<i>BRAFV600E</i>	-	+	Yes
6	Serrated	60	Not known	No	<i>BRAFV600E</i>	+	+	Yes
7	Serrated	83	Not known	No	<i>BRAFV600E</i>	-	+	Yes
8	Non-serrated	73	Not known	No	Wt	-	+	No
9	Non-serrated	72	Not known	No	Wt	-	+	No
10	Non-serrated	71	Not known	No	Wt	+	-	No
11	Non-serrated	53	Not known	Yes, Breast	Wt	-	+	No

Explanations: wt = wild type for both *KRAS* and *BRAF* mutations; - = negative; + = positive

Table 7. Mutation status of *BRAF* and *KRAS* according to the promoter methylation status of *hMLH1* and *MGMT* in serrated and non-serrated cancers

		<i>hMLH1</i> methylation				<i>MGMT</i> methylation			
		Yes		No		Yes		No	
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	p-value
Serrated adeno- carcinoma	<i>BRAF</i>V600E	10	10(100)	0(0.0)	0.001	11	10(90.9)	1(9.1)	0.019
	- wt <i>BRAF</i>	17	6(35.3)	11(64.7)		18	8(44.4)	10(55.6)	
	<i>KRAS</i> c12/13 or 59/61	13	5(38.5)	8(61.5)	0.034	14	5(35.5)	9(64.3)	0.005
	- wt <i>KRAS</i> (all)	14	11(78.6)	3(21.4)		15	13(86.7)	2(13.3)	
All non- serrated cancers	<i>BRAF</i> V600E	0	0	0	NA	0	0	0	NA
	- wt <i>BRAF</i>	43	10(23.3)	33(76.7)		43	18(41.9)	25(58.1)	
	<i>KRAS</i> 12/13 & 59/61	16	1(6.3)	15(93.8)	0.108	16	3(18.8)	13(81.3)	0.084
	- wt <i>KRAS</i> (all)	25	7(28.0)	18(72.0)		25	13(52.0)	12(48.0)	
Matched non- serrated cancers	<i>BRAF</i> V600E	0	0	0	NA	0	0	0	NA
	- wt <i>BRAF</i>	28	5(17.9)	23(82.1)		28	14(25.0)	14(25.0)	
	<i>KRAS</i> 12/13 & 59/61	13	1(7.7)	12(92.3)	0.343	15	3(23.1)	10(76.9)	0.014
	- wt <i>KRAS</i> (all)	16	4(25.0)	12(75.0)		16	11(68.8)	5(31.2)	

Explanations: wt= wild type; NA= not applicable.

Table 8. 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 <i>MGMT/hMLH1</i>	All N	MGMT expression*		<i>p</i> value (Fisher)	All N	hMLH1 expression		<i>p</i> value (Fisher)
			Positive or focal positivity*	Negative**			Positive	Negative	
All cases	Unmethylated	45	45	0	<0.0001	52	48	4	0.086
	Unmethylated&methylated	27	21	6		25	21	4	
	Methylated	11	5	6		5	3	2	
Serrated CRC	Unmethylated	13	13	0	0.041	13	12	1	0.186
	Unmethylated&methylated	17	13	4		14	11	3	
	Methylated	4	2	2		4	2	2	
Matched controls	Unmethylated	16	16	0	0.013	24	21	3	0.553
	Unmethylated&methylated	6	4	2		4	3	1	
	Methylated	6	3	3		1	1	0	

Explanations: *= For MGMT immunoreaction, tumour tissue presenting over 10% of positive cells was considered positive.

Table 9. The distribution of serrated and non-serrated cancers according to Jass classification.

	Putative Jass group						
	Group 1	Group 2	Group 3	Group 4	Group 5	Unclassified	All
	MSI-H and <i>BRAF</i> V600E mutation	MSS/MSI-L and <i>BRAF</i> V600E mutation	MSS/MSI-L and <i>KRAS</i> mutation	MSS/MSI-L, <i>wtKRAS</i> and <i>wtBRAF</i>	MSI-H, <i>wtKRAS</i> and <i>wtBRAF</i>	MSI-H and <i>KRAS</i> mutation	
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N
Serrated CRC	5 (14.7)	7 (20.6)	15 (44.1)	5 (14.8)	1* (2.9)	1 (2.9)	34
Non-serrated CRC	0 (0)	0 (0)	15 (37.5)	21 (52.5)	4 (10)	0 (0)	40

Explanations: $p < 0.0001$, exact contingency table; wt= wild type. The groups 1-5 follow the original categorisation of Jass⁶ with the exception of CIMP status and the methylation status of hMLH1 and MGMT that are not included in the definitions of the groups. *= Single case of serrated adenocarcinoma, bearing no evidence of HNPCC, is also illustrated in Figure 1G,H, and its clinical characteristics are shown in Table 6.

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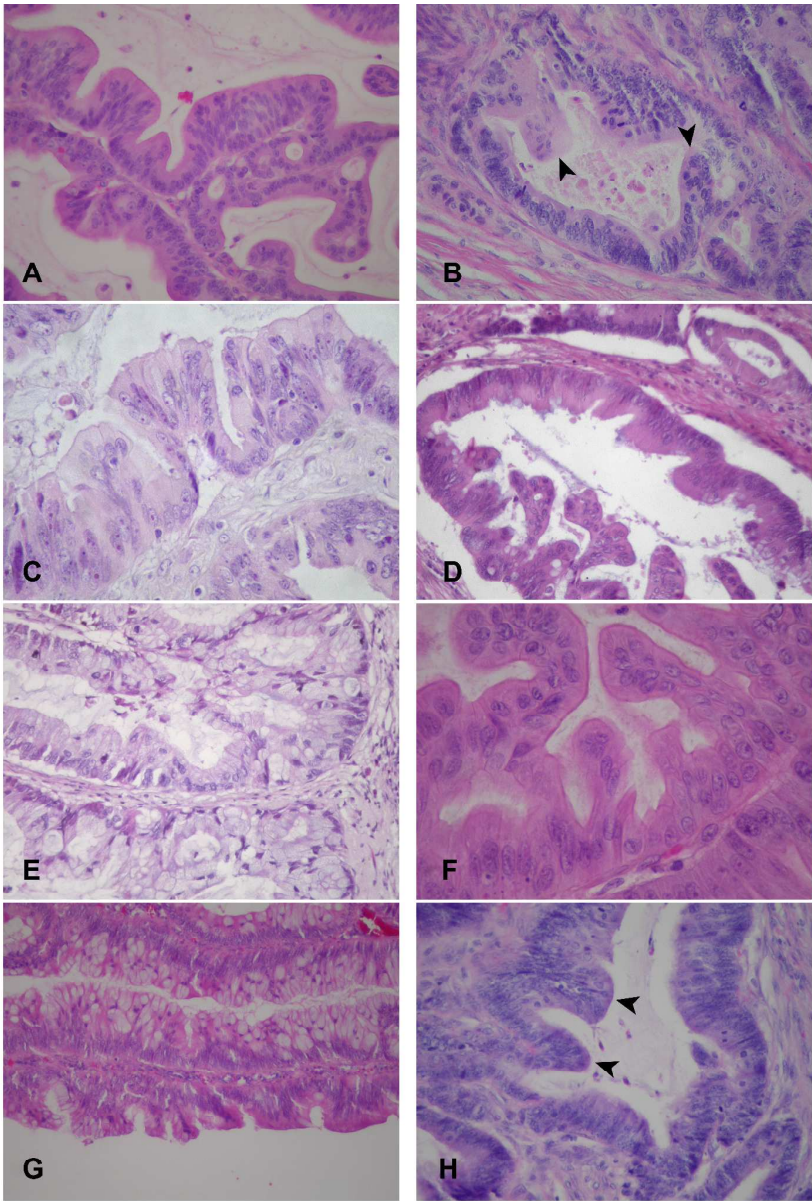
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For Peer Review



189x260mm (600 x 600 DPI)

FIGURE LEGENDS

Figure 1. Representative histological sections of serrated adenocarcinomas in regard to *KRAS*, or *BRAF* mutations and MSI status. A-C represent cases of serrated adenocarcinomas with *BRAFV600E* mutations, D-F serrated adenocarcinomas with *KRAS* c12 mutations, and G-H serrated adenoma and adenocarcinoma wild type for *BRAF* and *KRAS*, but representing with MSI-H.

(A) A case of serrated adenocarcinoma of the proximal colon harbouring *BRAFV600E* mutation and *MSI-H*. This case is well differentiated and mucin producing. The serrated epithelial folding is conspicuous.

(B) Another case of serrated adenocarcinoma of the proximal colon harbouring *BRAFV600E* mutation and *MSI-H*, initially diagnosed as conventional adenocarcinoma. Serrated epithelial folding is less conspicuous, but present (arrowheads).

(C) A case of MSS serrated adenocarcinoma of the proximal colon representing with *BRAFV600E* mutation.

(D) A case of MSS serrated adenocarcinoma of the proximal colon harbouring *KRAS* mutation. The serrations are apparent.

(E) A case of serrated adenocarcinoma of the proximal colon harbouring *KRAS* mutation and MSS/*MSI-L*. There is abundant clear cytoplasm, preserved polarity and luminal serration.

(F) A case of serrated adenocarcinoma of the proximal colon harbouring *KRAS* mutation and *MSI-H*. There is a prominent serrated pattern with epithelial tufting towards the lumen.

(G) A case of traditional serrated adenoma of the proximal colon. This tumour was wild type for both *KRAS* and *BRAF*, and *MSI-H*. The patient was 78 years old. There was no evidence of HNPCC.

(H) A case of serrated adenocarcinoma adjacent to traditional serrated adenoma represented in (G). The tumour shows preserved serrations (arrowheads).