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SHORT REPORT

Multiple APC mutations in sporadic flat colorectal adenomas

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Adenomas are established pre-malignant lesions in colorectal carcinogenesis. To date the adenoma–carcinoma sequence for the development of colorectal carcinoma (CRC) has been based largely on molecular data of exophytic, polypoid-type adenomas. Subsequently, a different type of adenoma has been identified: the flat adenoma, so called for its flat, non-exophytic appearance, making it less likely to be detected during conventional endoscopy. However, due to technological advances in endoscopic methods, flat-type adenomas can now frequently be detected and are no longer considered rare colorectal lesions. The phenotype of flat colorectal adenomas differs macroscopically and histologically from exophytic adenomas. Flat colorectal adenomas, as a rule, are tubular structures often revealing high-grade dysplasia, irrespective of the size or villous component. Flat adenomas have also been recognised as pre-cancerous lesions in gastric cancer. Unlike the wealth of clinical and molecular information available for polypoid (exophytic) adenomas, molecular profiles of flat-type lesions have not yet been characterised systematically and the full clinical significance hereof realised. Previous molecular investigation of the K-*ras* gene in flat colorectal adenomas suggests a distinct pathway in their development. In this study, mutation analysis of the adenomatous polyposis coli (*APC*) gene using the protein truncation test (PTT) in 20 flat colorectal adenomas in a selected group of 16 patients without hereditary predisposition to colorectal cancer, revealed double truncations of the *APC* gene in four adenomas. In one of these adenomas a third mutation was detected by DNA sequence analysis.

Keywords: sporadic flat colorectal adenomas; multiple *APC* mutations; protein truncation test

Introduction

Adenomas are frequently detected in patients with hereditary CRC syndromes and can be used as a clinical diagnostic marker in at-risk individuals.[1,2] A germ line mutation in either the adenomatous polyposis coli (*APC*) gene[3] or one of four mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1* or *hPMS2*)[4] renders an individual a greater lifetime risk of developing colorectal cancer than that of the general population. Somatic mutations in the APC and mismatch repair genes are ubiquitous in colorectal carcinomas.[4–7] Flat colorectal adenomas have previously been detected in individuals with and without hereditary colorectal

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cancer predisposition.[8–10] Flat colorectal adenomas are described as pre-malignant lesions with subtle morphological differentiation limited to the submucosa and no or limited protrusion into the colorectal lumen.[11] The malignant properties are evident during histological examination and little is known about the genetic changes that predispose to the development of the flat, rather than exophytic, appearance. This study is based on four flat adenomas obtained from four Swedish patients with a concomitant colorectal tumour. The *APC* gene mutated in the hereditary colorectal cancer syndrome, familial adenomatous polyposis coli (FAP), was analysed as a candidate causative gene in the aetiology of flat colorectal adenomas.

Materials and Methods

The patients in this study were referred for endoscopic examination to the Endoscopy Unit of the Karolinska Hospital, Stockholm, Sweden. The indications for endoscopy included change in stool habits, abdominal pain, anaemia, rectal bleeding and occult $FeHb^+$, previous excision of adenoma, CRC and concomitant colorectal carcinoma. They had no family history of colorectal cancer. Flat colorectal adenomas detected were biopsied and frozen prior to DNA extraction. Clinical characteristics are summarised in Table 1.

Genomic DNA was extracted from frozen tissue samples (cut into small pieces or homogenised with a Polytron). Lysis buffer (75 mM NaCl, 24 mM EDTA), 1–2 mg proteinase K and 1–2% SDS were added to the cells and the suspension incubated at room temperature overnight or for 4 h at 55°C. Following phenol and chloroform extraction, the DNA was precipitated with NH_4Ac (7.5 M)/propanol, washed in ethanol and dissolved in TE^{-4} (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). A 50 µl PCR reaction contained 100 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (TaKaRa Biomedicals, Otsu, Shiga, Japan), 0.3 U *Taq* polymerase (TaKaRa Biomedicals), 50 pmol of each primer, in 1 × PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCL) (TaKaRa Biomedicals). The forward primers used were previously published by Prosser *et al*,[12] whilst reverse primers G2, J2, Q2 and U2 for segments 2, 3, 4, and 5, respectively, have been published by Groden *et al*.[3] Reactions were overlaid with mineral oil and subjected to thermocycling as follows: initial heat denaturation at 95°C (5 min); [95°C denaturation (1 min), annealing temperature (2 min), 72°C extension (1 min)] for 35 cycles and final extension at 72°C (5 min) in a Hybaid® thermocycler. Annealing temperatures for segments 2, 3, 4 and 5 were 66°C, 65°C, 62°C and 62°C, respectively.

Radioactive *in vitro* transcription/translation of PCR generated fragments was performed using the TNT T7 Quick Coupled Transcription/Translation System® (Promega, Southampton, UK) according to the manufacturer's instructions. In brief, the reaction contained 2–5 µl of PCR amplified product, 8 µl of the transcription/translation mixture, 5 µCi translation grade ^{35}S L-methionine (Amersham Life Sciences, Cleveland, OH, USA) and TE^{-4} (10 mM Tris-HCl pH 7.7, 0.1 mM EDTA) to a final volume of 15 µl. The reaction was incubated at 30°C for 90 min in a thermal cycler. Resolution of the transcription/translation generated polypeptides was accomplished by SDS polyacrylamide gel electrophoresis as described in the Technical Bulletin No 126 (Promega), with the exception that the gels were run at constant voltage (200–300 volts) for 2–3 hours. Following fixation in 10% acetic acid/20% methanol, gels were dried and exposed to X-OMAT™ scientific imaging film (Kodak). DNA sequence analysis of PCR products was performed on an Applied Biosystems (AB373) DNA sequencer using fluorescent dideoxynucleotides. Alternatively, radioactive-based sequence analysis was conducted using the T7 Sequenase Version 2.0 PCR Product Sequencing Kit (Amersham Life Sciences) according to the manufacturer's instructions incorporating ^{35}S-dATP. Primers used for sequence analysis have been previously published.[3,13]

Results

DNA analysis of exon 15 of the *APC* gene was performed using polymerase chain reaction amplification in four overlapping fragments (segment 2–5), followed by an *in vitro* synthesised protein assay (PTT).[12] In four samples obtained from the 20 single flat adenoma in four different individuals, two different truncated variants each in the 5' region of exon 15,

Table 1 Clinical characteristics of patients with flat adenomas

				Flat adenoma		
Sample no.	*Age (yrs)*	*Gender*	*Clinical history*	*Diameter (mm)*	*Location in colon*	*Histology*
1[a]	54	M	Fatigue, changed bowel habits, episodes of diarrhoea	6	transverse	[b]TA LGD
2[c]	70	M	Anaemia	8	ascendens	[b]TA LGD
3[a]	79	F	Previous sigmoideum cancer, changed bowel habits	3	ascendens	[b]TA LGD
4[c]	73	F	Anaemia, changed bowel habits	16	transverse	[b]TA LGD

[a]Tubulovillous adenoma at the right flexure (sample no 1) and caecum (sample no 3); [b]Tubular adenoma with low grade dysplasia; [c]Adenocarcinomas identified in caecum (sample no 2) and transverse (sample no 4) colon.

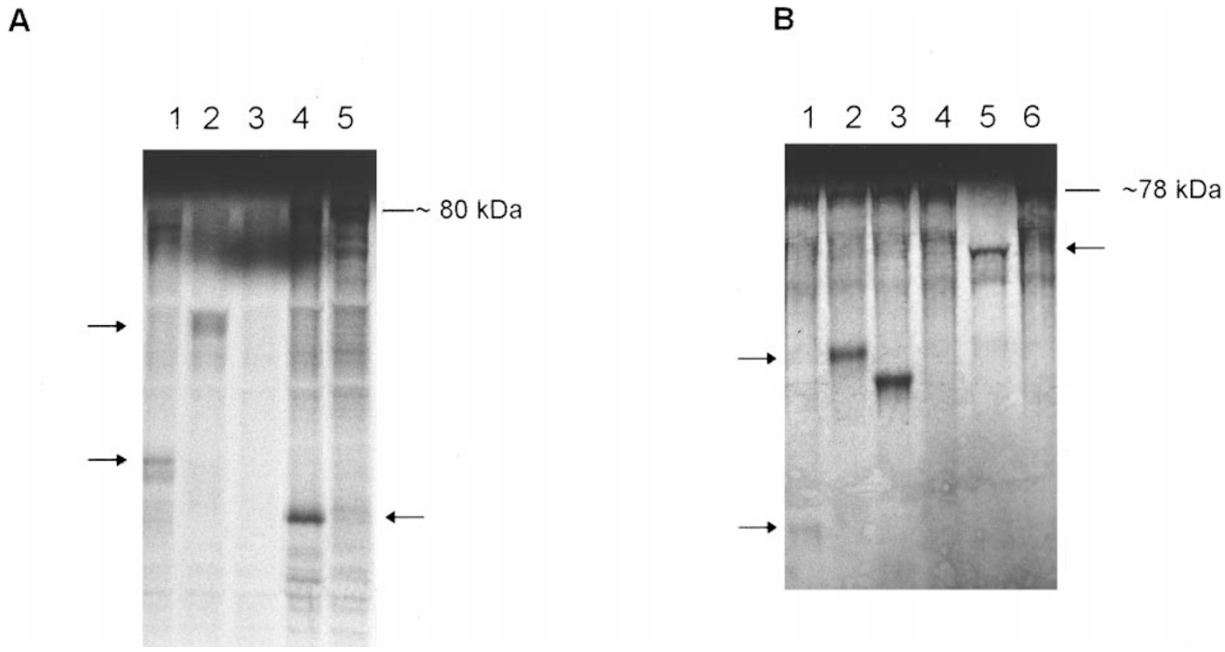

Figure 1 *Autoradiogram showing PTT variants in segment 2 (***A***) and segment 3 (***B***) of exon 15 of the* APC *gene. Non-specific transcripts may be the consequence of translation initiation at internal ATG sites.* **A** *Lanes 1, 2, 4: truncated variants in sample nos 1, 4 and 3, respectively; lanes 3, 5: negative controls. Full length segment 2 transcript about 80 kDa.* **B** *Lanes 1, 2, 5: truncated variants in sample nos 1, 2 and 4, respectively – note the apparent absence of the normal length transcript in sample no 4 (lane 5); lane 4: PTT analysis on blood DNA of sample no 2 lacking the truncated variant observed in the flat colorectal adenoma; lanes 3 positive control and lane 6 negative control. Full length segment 3 transcript about 78 kDa.*

which encompasses the mutation cluster region,[6] were detected (Figure 1). In four of the remaining 16 adenomas single *APC* truncations have been detected (data submitted elsewhere). None of the PTT variants were present in peripheral blood DNA of the respective patients, verifying somatic origin of variants (Figure 1). Upon radioactive and fluorescent-based sequencing of samples demonstrating truncated variants, an additional missense mutation was identified in a single sample (sample no 3), resulting in the detection of three mutations in this sample (Table 2). Unfortunately, the PTT variants in segment 2 could not be characterised using DNA sequence analysis due to the limited amount of DNA available for analysis.

Table 2 Characterisation of mutations in colonic flat adenomas displaying double APC truncations

Sample no.	*Amplicon*	*Codon*	*Nucleotide change*	*Mutation type and result*
1	Segment 2	?	?	?
	Segment 3	1309	GGA AAG ATT→ GGT T	Frameshift deletion, termination at codon 1314
2	Segment 2	?	?	?
	Segment 3	1451	CGA→TGA	Nonsense, Arg→stop
3	Segment 2	?	?	?
	Segment 3	1451	CGA→TGA	Nonsense, Arg→stop
		1492	TTT→TAT	Missense, Phe→Tyr
4	Segment 2	?	?	?
	Segment 3	1557	AAA ACT ATT→ AAA AAC TAT T	Frameshift insertion, termination at codon 1559

?: uncharacterised.

Discussion

In a recent report by Olschwang *et al*[14] double APC protein truncations were reported in two flat colorectal adenomas, which they ascribed to biallelic inactivation. Powell *et al*[15] also detected double *APC* mutations in each of nine samples. Another single adenoma with three *APC* mutations revealed multifocal origin of development at histopathological examination. No distinction was made, however, regarding the luminal protrusion of the adenoma (flat or exophytic). Mutations resulting in the creation of premature stop codons can be harboured on a single allele within a cell line, in which case the more downstream stop codons are created, they will have no effect on the truncated protein structure, since translation is terminated at the first stop codon encountered.

In three cases Powell *et al*[15] confirmed biallelic inactivation by inclusion of the two variant loci in the same PCR amplification, followed by cloning and sequence analysis. However, in this study, it was not possible to determine the allelic location (*cis* or *trans*) of the double truncations because of the inability to characterise all the variants. Theoretically, the presumption of biallelic inactivation cannot accurately be confirmed by inclusion of the two variant loci in the same PCR amplification in these samples, since during PCR cycling not all strands are fully extended due to the length of the amplicons required by the distance between the mutations. Upon annealing of the incomplete fragments on the opposite strand and subsequent elongation, it may appear that the variants are on separate strands, whilst in the genomic context they are not. When the loci are in extremely close proximity, as was the case with two samples (Ca-10 and Ca-14) in the study by Powell *et al*,[15] it will be unlikely for a detectable proportion of amplicons to be hybrids.

Assuming biallelic inactivation, the theory that inactivation of the second allele of *APC* occurs early in adenoma development[15,16] is supported by the double truncations observed in these small flat adenomas. However, it is possible that the mutations arose in different cell lines, implicating a polyclonal origin, especially since more than two mutations can be present in the same lesion.[15] Ming and Yu[17] demonstrated multifocal development of flat adenomas, as opposed to clonal expansion of exophytic adenomas, on histopathological examination. More than one initiating clone in close proximity to each other can develop into the same adenoma, provided that the combined accumulated mutations originate in more than one cell line, as postulated by Merritt *et al*[18] based on findings in the murine model of FAP, ApcMin. However, the possibility of a multi-hit event on the same DNA strand cannot be ruled out since a second and even third mutational event can occur in the same molecule.

The full-length transcripts of segments 2 (nt 1972–4152) and 3 (nt 3295–5127) of exon 15 were present in all the samples. However, a reduction in intensity of the normal allele could be observed in segment 3 of adenoma number 4 (Figure 1B, lane 5), indicating partial loss of heterozygosity at the corresponding site on the normal allele in this segment. According to Bjerknes *et al*[19] the adenomatous crypts in the colorectum consist of a mixture of normal and mutant cell lines. In addition, 'contamination' with surrounding normal mucosa is almost inevitable when sampling such small lesions. This may lead to apparent 'dilution' of aberrant clones, impeding the demonstration of loss of heterozygosity or the detection of mutations in dysplastic tissue. The frequency of biallelic inactivation can thus be underestimated in this study since loss of the second (normal) allele is often observed in sporadic colorectal tumours.[20]

In this study we have identified multiple somatic *APC* mutations in flat colorectal adenomas. The degree of dysplasia, the size of the adenoma or the presence of a villous component did not appear to reflect the frequency of mutations (Tables 1 and 2). Although several hypotheses on the adenoma–carcinoma pathway for colorectal cancer exist, the initiating event in the formation and progression of these flat adenomas is shared with polypoid (exophytic) adenomas. It can therefore be speculated that factors other than a mutation in the *APC* gene determine polypoid growth. Systematic molecular analysis of other genes implicated in human cancer at different progressive stages of flat adenoma development will shed light on the transformation process of flat adenoma to adenocarcinoma.

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References

1 Muto T, Bussey HJR, Morson BC: The evolution of cancer of the colon and rectum. *Cancer* 1975; **36**: 2251–2270.

2 Muller H, Scott R, Weber W, Meier R: Colorectal cancer: lessons for genetic counselling and care for families. *Clin Genet* 1994; **46**: 106–114.
3 Groden J, Thliveris A, Samowitz W *et al*: Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 1991; **66**: 589–600.
4 Papadopoulos N, Lindblom A: Molecular basis of HNPCC: mutations of *MMR* genes. *Hum Mut* 1997; **10**: 89–99.
5 Miyaki M, Konishi M, Kikuch-Yanoshita R *et al*: Characteristics of somatic mutation of the Adenomatous Polyposis Coli gene in colorectal tumours. *Cancer Res.* 1994; **54**: 3011–3020.
6 Miyoshi Y, Nagase H, Ando H *et al*: Somatic mutations in the *APC* gene in colorectal tumours: mutation cluster region in the *APC* gene. *Hum Mol Genet* 1992; **1**: 229–233.
7 Wu Y, Nystrom-Lahti M, Osinga J *et al*: MSH2 and MLH1 mutations in sporadic replication error positive colorectal carcinoma as assessed by two-dimensional DNA electrophoresis. *Genes, Chromos, Cancer* 1997; **18**: 269–278.
8 Minamoto T, Sawaguchi K, Ohta T, Itoh T, Mai M: Superficial-type adenomas and adenocarcinomas of the colon and rectum: a comparative morphological study. *Gastroenterology* 1994; **106**: 1436–1443.
9 Watanabe T, Muto T, Sawada T, Miyaki M: Flat adenoma as a precursor of colorectal carcinoma in hereditary nonpolyposis colorectal carcinoma. *Cancer* 1996; **77**: 627–634.
10 Kubota O, Kino I: Minute adenomas of the depressed type in familial adenomatous polyposis of the colon. *Cancer* 1993; **72**: 1159–1164.
11 Muto T, Kamiya J, Sawada T *et al*: Small 'flat adenoma' of the large bowel with special reference to its clinopathologic features. *Dis Colon Rectum* 1985; **28**: 847–851.
12 Prosser J, Condie A, Wright M *et al*: *APC* mutation analysis by chemical cleavage of mismatch and a protein truncation assay in familial adenomatous polyposis. *Br J Cancer* 1994; **70**: 841–846.
13 Miyoshi Y, Ando H, Nagase H *et al*: Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients. *Proc Natl Acad Sci USA* 1992; **89**: 4452–4456.
14 Olschwang S, Slezak P, Roze M *et al*: Somatically acquired genetic alterations in flat colorectal neoplasms. *Int J Cancer* 1998; **77**: 366–369.
15 Powell S, Zilz N, Beazer-Barclay Y *et al*: *APC* mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**: 235–237.
16 Cunningham C, Dunlop MG: Genetics of colorectal cancer. *Br Med J Bull* 1994; **50**: 640–655.
17 Ming S, Yu P: Histogenesis of experimental colonic carcinomas. In: Rozen P, Reich CB, Winawer SJ (eds). *Large Bowel Cancer: Policy, Prevention, Research and Treatment. Front Gastrointest Res*, Basel, Karger, 1991; **18**: 200–224.
18 Merritt AJ, Gould KA, Dove WF: Polyclonal structure of intestinal adenomas in ApcMin/$^{+}$ mice with concomitant loss of Apc^{+} from all tumor lineages. *Proc Natl Acad Sci USA* 1997; **94**: 13927–13931.
19 Bjerknes M, Cheng H, Kim H, Schnitzler M, Gallinger S: Clonality of dysplastic epithelium in colorectal adenomas from adenomatous polyposis patients. *Cancer Res* 1997; **57**: 355–361.
20 Vogelstein B, Fearon ER, Hamilton SR *et al*: Genetic alterations during colorectal tumour development. *N Engl J Med* 1988; **319**: 525–532.