K-ras2 activation and genome instability increase proliferation and size of FAP adenomas

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The possible role of K-ras2 mutations and aneuploidy toward increase of proliferation and adenoma size in Familial Adenomatous Polyposis (FAP) adenomas is not known. The present study addresses these issues by investigating 147 colorectal adenomas obtained from four FAP patients. The majority of adenomas had size lower than or equal to 10 mm (86%), low grade dysplasia (63%), and were preferentially located in the right colon (60%). Normal mucosa samples were obtained from 19 healthy donors. Three synchronous adenocarcinomas were also investigated. K-ras2 mutation spectrum was analysed by PCR and Sequence Specific Oligonucleotide (SSO) hybridization, while flow cytometry (FCM) was used for evaluating degree of DNA ploidy and S-phase fraction. Overall, incidences of K-ras2 mutations, DNA aneuploidy and high S-phase values (>7.2%) were 6.6%, 5.4% and 10.5%, respectively. In particular, among the adenomas with size lower than 5 mm, K-ras2 mutation and DNA aneuploidy frequencies were only slightly above 1%. Statistically significant correlations were found between K-ras2 and size, DNA ploidy and size and K-ras2 and S-phase ($p < 0.001$). In particular, among the wild type K-ras2 adenomas, high S-phase values were detected in 8% of the cases versus 57% among the K-ras2 mutated adenomas ($p = 0.0005$). The present series of FAP adenomas indicates that K-ras2 activation and gross genomic changes play a role toward a proliferative gain and tumour growth in size.

Keywords: FAP, colorectal adenomas, ras gene, aneuploidy, proliferation

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1. Introduction

Familial Adenomatous Polyposis (FAP) syndrome, characterized by hundreds of adenomatous polyps of different size and degree of dysplasia, is a highly penetrant autosomal dominant disorder associated with the germ line mutation of the APC gene [17,22,24,33]. If colectomy is not performed, FAP patients have a high probability of developing one or more colorectal cancers. Colorectal cancer development in FAP and sporadic patients is considered to be due to the accumulation of several genetic events including inactivation of the APC gene, mutations of K-ras2, p53 and DCC, and LOH of several chromosomes as recently indicated using Comparative Genomic Hybridization [7,9, 20,27,37,42]. K-ras2 mutations in FAP colorectal tumors were suggested to play a secondary role, since the mutation incidence in the small FAP adenomas is low (lower than 10%) while the second APC allele is already mutated in about 50% of the cases [2,20,25,27]. K-ras2 mutation incidence in colorectal tumours, more frequent in the polyp forming cancer, is up to about 50% in both sporadic adenomas and adenocarcinomas [9,42], while in the non-neoplastic aberrant crypt foci considered to be early precursors of adenomas was detected up to 85% [35,36,41,44].

Recently, new functions of the Ras proteins were suggested with the discovery of a plethora of new effector pathways and different cellular mechanisms in-

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cluding phosphorylation of transcription factors in the MAP kinase cascade [6] and association with proliferation [1,4,40], apoptosis [8,23,43], cytoskeleton organization [19,34], chromosome stability and mitotic integrity [18,32]. We have previously shown by DNA flow cytometry (FCM) that human sporadic colorectal adenomas were characterized by an incidence of DNA aneuploidy of about 30% mainly distributed in the near-diploid region [11,14,16]. Aneuploidization mechanisms in sporadic colorectal adenomas were also suggested to be dependent from K-ras2 oncogene mutational activation while specific mutations (G $\rightarrow$ A transitions in codon 12) were associated to inhibition of proliferation [12,15].

Since literature data on the relationships among K-ras2 mutations, aneuploidy, proliferation, and growth in size in human colorectal adenomas from patients with the FAP syndrome are very scanty, we have decided to address these issues in the present study. In particular for a single patient, who underwent total colectomy, we mapped and analyzed a relatively large number of small adenomas (66% of a total of 123 adenomas had size lower than 5 mm).

2. Material and methods

2.1. Study population

The study was performed on 147 polyps (size range between 2 and 35 mm; mean $= 6.3 \pm 5.3$ mm) with a histological diagnosis of adenomas and 3 adenocarcinomas from 4 FAP patients (3 females, 1 male). 87 adenomas were located in the right colon (including caecum, ascending colon, hepatic flexure and transverse colon), and 60 in the left colon (including splenic flexure, descending and sigmoid colon, and rectum). Patients were 19–53 years old (mean = 31 years). Control samples were taken from the normal FAP mucosa (12 samples) and from 19 healthy donors.

2.2. Histological analysis and topographic selection

Polyps were divided into two specular parts by a central mid-saggital section. One specimen was fixed in 10% buffered formalin for 24 h, handled according to customary and histopathological diagnosis protocols and embedded in paraffin. The other specimen was used to provide fresh-frozen multiple samples for the FCM and PCR amplification. Histological diagnosis and grading were according to the World Health Organization criteria [21]. Dysplasia was divided in low grade, including mild and moderate, and high grade, corresponding to severe dyplasia. We obtained 93 low grade and 54 high grade dysplastic adenomas. Regarding dimensions, we had 84 polyps smaller than 5 mm, 43 polyps ranging from 5 to 10 mm, and 20 polyps larger than 10 mm in diameter (Table 1).

Table 1
Age, sex, site, size, and dysplasia in 147 adenomas from 4 FAP patients

Patients (sex[a]/age)	Site[b]	Size (mm)			Dysplasia[c]	
		< 5	$5 \leqslant S \leqslant 10$	> 10	LG	HG
TO1(F/19)	R	55	10	–	38	27
	L	26	23	9	33	25
PD1(M/53)	R	1	9	5	15	–
	L	–	–	–	–	–
NA1(F/23)	R	1	1	3	5	–
	L	1	–	–	1	–
NA2(F/29)	R	–	–	2	1	1
	L	–	–	1	–	1
Total	R	57	20	10	59	28
	L	27	23	10	34	26
	R + L	84	43	20	93	54
Frequency		57%	29%	14%	63%	37%

[a] M = male; F = female.
[b] R, right colon includes caecum, ascending colon, hepatic flexure, and transverse colon; L, left colon includes splenic flexure, descending colon, sigmoid colon, and rectum.
[c] LG = low grade dysplasia; HG = high grade dysplasia.

2.3. DNA flow cytometry

Tissue fragments were minced with scalpels for 1 to 2 minutes directly in the staining solution composed of 10 mM phosphate-buffer in isotonic saline, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.6% Nonidet P40 (v/v), 0.2% bovine serum albumin (w/v), and 10 mg/l of DAPI (Sigma Chemical Co., St. Louis, MO). Nuclei suspensions were measured by flow cytometry as previously detailed [15,16]. G_0–G_1 DNA content in adenomas was considered aneuploid in presence of an extra G_0–G_1 peak. Trout erythrocytes and individual-specific normal mucosa were also used as reference DNA standards. The degree of DNA aneuploidy (DI) was calculated as ratio of mean channel number of epithelial aneuploid G_0–G_1 peak to mean channel number of peak corresponding to normal mucosa and/or tissue infiltrating G_0–G_1 lymphocytes. The mean coefficient of variation (CV) was $3.3 \pm 0.7\%$. The S-phase fraction was evaluated on the whole samples using dedicated software (Phoenix Flow Systems). Aliquots of approximately 20 000 nuclei obtained from the same suspension were collected to perform K-ras2 mutation analysis.

2.4. K-ras2 analysis

Extracted DNA from peripheral blood lymphocytes from healthy donors were used as wild type K-ras2 codon 12 GGT-gly and codon 13 GGC-gly controls. Additionally, 6 cell line extracted DNAs were used as controls for known K-ras2 mutations as previously described [12]. FAP samples were collected in aliquots of 20 000 nuclei, stored at $-80°C$, and amplified by polymerase chain reaction, as previously detailed [12]. Oligonucleotide 20-mer panel (TIB MOLBIOL, Advanced Biotechnology Center, Genoa, Italy) for sequence specific oligonucleotide hybridization included K-ras2 codon 12 and 13 wild type sequences, all possible mutations of codon 12, and the AGC and GAC mutations of codon 13. The probes were 5′-end labeled by phosphorylation with [γ-^{32}P]ATP, according to the standard method [39]. Dot blot and hybridization were done as usual [15].

2.5. Statistical analysis

Contingency tables were used to evaluate the correlation among K-ras2 status, DNA index, S-phase fraction values and the other adenoma parameters (site, size, dysplasia). Statistical significance was evaluated by means of the Chi-square test, with the Yates correction when appropriate, and was set in advance at $p = 0.01$ [10].

3. Results

Figure 1 shows two examples of the flow cytometric analysis of DNA content of FAP adenoma samples: in Fig. 1A only one diploid peak is visible, in B an extra DNA-aneuploid peak in the near-triploid region was detected.

Table 1 summarizes the distribution of site, size and dysplasia of 147 adenomas belonging to 4 FAP patients. The first patient was investigated by analyzing a total of 123 adenomas whose mean size and SD were 5 ± 3 mm. Overall, the size of 57% of the adenomas was smaller than 5 mm, ranged between 5 and 10 mm in 29%, and was greater than 10 mm in 14% of the present series only. About 60% of the polyps were located in the right colon and 40% in the left. Regarding the grade dysplasia, 63% of the adenomas were low grade and 37% were high grade.

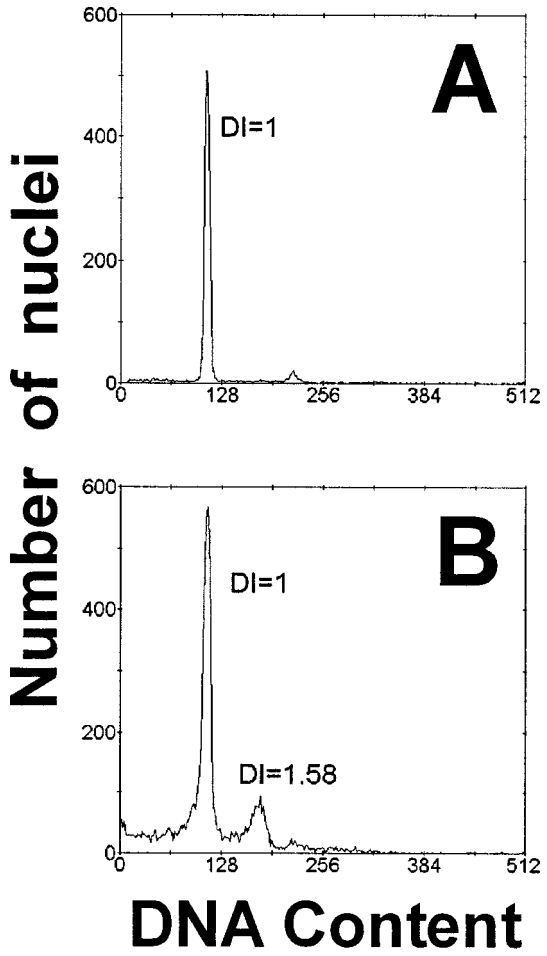

Fig. 1. Examples of DNA content analysis of two FAP adenoma samples by flow cytometry (see the text Result section).

Table 2

K-ras2 mutations, DNA aneuploidy, and high S-phase among 147 colorectal adenomas and 3 adenocarcinomas in 4 FAP patients

Patients	N. of tumours analyzed	Mean size (mm ± SD)	K-ras2 mutations	DNA aneuploidy	High-S[a]
Adenomas					
TO1	123	5 ± 3	2/113 (1.8%)	4/123 (3.2%)	9/121 (7.4%)
PD1	15	9.8 ± 4	5/15 (33%)	0/15 (0%)	4/15 (26.7%)
NA1	6	18.8 ± 14.7	0/6 (0%)	2/6 (33%)	2/6 (33%)
NA2	3	17 ± 2.9	2/3 (67%)	2/3 (67%)	0/1 (0%)
Total tumours	147	6.3 ± 5.3	9/137 (6.6%)	8/147 (5.4%)	15/143 (10.5%)
Synchronous adenocarcinomas					
PD1	2		0/2	0/2	0/2
NA2	1		0/1	1/1	1/1

[a] High S-phase threshold value was 7.2% as determined on the basis of the mean S-phase value (5.1 ± 0.7%) added of 3SD determined in multiple samples of normal mucosa obtained from 19 healthy donors. S-phase was rejected in 4 cases (2.7%) due to high CV and background values.

Table 3

Adenoma site/size, dysplasia, specific K-ras2 mutations, DNA Index, and S-phase fraction values of 9 K-ras2 mutated FAP colorectal adenomas

Patient	Adenoma site/size[a]	Dysplasia[b]	K-ras[c] mutations	DNA Index[d] (CV)	S-phase fraction (%)
TO1	R/10	LG	GAC	1.0 (3.0%)	3.2
	R/4.2	LG	TGT	1.0 (2.4%)	5.2
PD1	R/15	LG	TGT	1.0 (2.2%)	7.3
	R/10	LG	TGT	1.0 (2.7%)	7.6
	R/17.5	LG	TGT	1.0 (2.4%)	5.7
	R/13	LG	TGT	1.0 (3.0%)	8.3
	R/8	LG	AGC	1.0 (2.5%)	9.1
NA2	R/15	HG	GAT	1.08 (4.0%)	–
	L/20	HG	GAT	1.58 (4.2%)	–

[a] Site was right (R) or left (L) colon: R includes caecum, ascending colon, hepatic flexure, transverse colon; L includes splenic flexure, descending colon, sigmoid colon and rectum. Size was indicated by approximate diameter in mm.
[b] LG = low grade dysplasia; HG = high grade dysplasia.
[c] Wild type codon 12 and 13 were respectively GGT and GGC.
[d] DNA Index $\neq$ 1 was for DNA aneuploidy (DI = 1 is for DNA diploidy) as detected by flow cytometry. CV stays for Coefficient of Variation of the G_0/G_1 DNA diploid/aneuploid peaks. Mean CV obtained from multiple samples of histologically proven normal mucosa from 19 healthy donors was 2.98 ± 0.39. In these experimental conditions DNA aneuploid peaks are detected at about ±4% DNA changes in presence of at least 5–10% DNA aneuploid cells.

Table 2 shows that the overall incidence of K-ras2 mutations, DNA aneuploidy, and high S-phase values among the 147 adenomas investigated were 6.6% (9/137), 5.4% (8/147), and 10.5% (15/143), respectively. S-phase fraction values were considered high when greater than the threshold value of 7.2%. This threshold value was obtained from a series of multiple samples of normal mucosa obtained from 19 healthy donors after addition of 3 Standard Deviations.

DNA aneuploid values (with DNA Index, DI $\neq$ 1) were near-diploid (DI $\leqslant$ 1.3) in 5 out of 8 cases. Two patients had 3 synchronous adenocarcinomas. In one patient (PD1), neither of the two adenocarcinomas showed K-ras2, DNA ploidy nor S-phase abnormalities. For the patient NA2, DNA aneuploidy and high S-phase, but no K-ras2 mutation, were detected in the adenocarcinoma investigated. One may observe that K-ras2 mutation and DNA aneuploidy incidences among

Table 4
K-ras2 status, DNA ploidy, S-phase versus site, size, dysplasia, and K-ras2 status versus DNA ploidy and S-phase in 147 adenomas from 4 FAP patients

	Site[a]		Size (mm)			Dysplasia[b]	
	R	L	< 5	$5 \leqslant S \leqslant 10$	> 10	LG	HG
K-ras2 status							
wt	74	54	78	38	12	79	49
mutated	8	1	1	3	5	7	2
				$p = 0.0001$			
DNA Index[c]							
DI $= 1$	83	56	83	40	16	90	49
DI $\neq 1$	4	4	1	3	4	3	5
				$p = 0.0003$			
S-phase[d]							
low-S	76	52	76	38	14	83	45
high-S	9	6	7	4	4	10	5

	DNA Index[c]		S-phase[d]	
	DI $= 1$	DI $\neq 1$	low-S	high-S
K-ras2 status				
wt	122	6	116	10 (8%)
mutated	7	2	3	4 (57%)
	$p = 0.15$		$p = 0.0005$	

[a] Site was right (R) or left (L) colon: R includes caecum, ascending colon, hepatic flexure, and transverse colon; L includes splenic flexure, descending colon, sigmoid colon, and rectum.
[b] LG = low grade dysplasia; HG = high grade dysplasia.
[c] DNA Index (DI) $\neq$ 1 was for DNA aneuploidy (DI $=$ 1 is for DNA diploidy) as detected by multiparametric flow cutometry.
[d] High S-phase threshold value was 7.2% as determined on the basis of the mean S-phase value (5.1 $\pm$ 0.7%) added of 3SD determined in 19 samples of normal mucosa obtained from healthy donors. S-phase was rejected in 4 cases (2.7%) due to high CV and background values.

the individual patients ranged widely (from 0 to 67%) similarly to high S-phase values (from 0 to 33%). Normal FAP mucosa was never found with abnormal values of these three parameters.

The specific K-ras2 mutations, DI and S-phase values of the 9 K-ras2 mutated adenomas are reported in Table 3 together with site, size, and grade of dysplasia. It is interesting to note the presence of two different K-ras2 mutations in patients TO1 and PD1. The mutations, 5 G-T tranversions and 4 G-A transitions, were mainly detected in low grade dysplastic adenomas ($7/9 = 78\%$). Moreover, 8 out of 9 K-ras2 mutated adenomas were located in the right colon. The S-phase fraction exceeded the threshold value of 7.2% in 4 out of 7 mutated adenomas. It should be noted that only 2 among the 9 K-ras2 mutated adenomas were DNA aneuploid.

Table 4 shows K-ras2 status, DNA ploidy and S-phase fraction versus site, size and dysplasia, and K-ras2 status versus DNA Index and S-phase fraction. It is to be noted that there is a progressive increase of the number of K-ras2 mutated adenomas, as well as of the DNA aneuploid adenomas, with increase of size ($p = 0.0001$ and $p = 0.003$, respectively). K-ras2 was not correlated with DNA Index but showed a statistically significant correlation with S-phase fraction, i.e., mutated adenomas had high S-phase in 57% of the cases versus 8% for the wild type ($p = 0.0005$).

4. Discussion

Human colorectal adenomas arising in Familial Adenomatous Polyposis (FAP) syndrome patients are associated to the germ line mutation of the APC oncosuppressor gene [17,22,24,33] and to a somatic alteration of the second normal allele [20,28,29,31] according to the two-hits hypothesis of Knudson [26]. While

the initiating events of the human FAP syndrome are well established, later genotypic events associated with tumour progression are not yet well known. We have presently studied the role of K-ras2 activation and aneuploidy in relationship with a possible proliferative gain and increased growth in size using in vast majority small adenomas (86% were smaller than 10 mm). Analyses were performed on fresh-frozen material by taking multiple specimens from every lesion. One patient was studied in greater details by mapping and investigating 123 adenomas. Flow cytometry was used to measure relative DNA content to provide the degree of DNA aneuploidy (also known as DNA Index, DI) and the S-phase fraction. The same samples were also investigated by sequence specific oligonucleotide hybridization in codon 12 and 13 to provide the K-ras2 mutation spectrum.

Normal FAP mucosa and mucosa from healthy donors were investigated for comparison and were never found to give abnormal values of K-ras2 status, DNA ploidy and S-phase fraction.

K-ras2 mutation incidence among the present series of 147 adenomas was about 7%. K-ras2 mutations and size were strongly correlated: among small adenomas (size $<$ 5 mm) only about 1% ($1/79$) were mutated versus 30% of the large ones (size $>$ 10 mm). Moreover, 57% among the K-ras2 mutated adenomas (versus 8% of wild type K-ras2) showed S-phase fraction greater than the threshold value of 7.2% ($p = 0.0005$).

Thus, the present study provides experimental evidence that neither K-ras2 activation nor DNA aneuploidy are a characteristic of small adenomas among FAP syndrome patients. Previous studies had already shown that K-ras2 oncogene activation appears associated to a more aggressive phenotype in larger FAP adenomas [2,9,20,27,42]. The positive relationship of K-ras2 activation and a proliferative gain in FAP patients, however, was not previously reported using the present techniques. This finding is different for human sporadic colorectal adenomas where K-ras2 mutations (and, in particular, G→A transitions) were associated to inhibition of proliferation [12]. It is likely that this difference arises from the fact that FAP adenomas are in presence of alteration of both the APC alleles. In addition, the fact that K-ras2 mutations were already detected up to about 85% in human colorectal aberrant crypt foci in sporadic cancer patients [35,36,38,41,44], suggests that K-ras2 activated lesions without APC inactivation have little potential to progress.

Interestingly and newly in comparison with previous studies, we have also detected a statistically significant correlation between DNA aneuploidy and size ($p = 0.003$). DNA aneuploidy incidence was, in fact, only about 1% in the small adenoma class (size $<$ 5 mm) and 20% for the large adenomas (size $>$ 10 mm).

Thus, loss of chromosome stability appears to be an acquired mechanism toward an increase in size and potentially malignant characteristics. DNA aneuploidy was reported to be absent in aberrant crypt foci in both FAP and sporadic patients [30] and to be above 20% among human sporadic colorectal adenomas with different sizes with mild-moderate dysplasia and up to 50% and 70% in respectively severe dysplastic adenomas and adenomas with early cancer [14]. The data in literature regarding aneuploidy in FAP tumours are quite few. DNA aneuploid subclones detected in the present series of FAP adenomas were near-diploid (DI $\leqslant$ 1.3) in about 63% of the cases. This finding is in agreement with our previous data in human sporadic adenomas in which aneuploid subclones were in the near-diploid range in about 70% of the cases [11,14].

Chromosome instability and aneuploidy appear to be dependent on very complex molecular mechanisms that still remain unknown. A possible link of aneuploidy with mutations of K-ras2 oncogene and p53 in human sporadic preneoplastic colorectal lesions has been recently reviewed [13]. Several experimental studies performed both *in vitro*, using mainly K-ras2 mutated transfected cell lines [18,32], and *in vivo*, using human colorectal sporadic adenomas of increasing size [12,15], suggest a possible role of K-ras2 gene mutations in affecting chromosome stability. In the present study, however, among 9 K-ras2 mutated FAP adenomas, only 2 were DNA aneuploid. The present findings suggest that K-ras2 activation in FAP adenomas cooperates mainly to alter the proliferative status without affecting the genome stability.

A possible role of K-ras2 activation to inhibit apoptosis, as previously suggested using *in vitro* colon cell lines [3] and human sporadic preneoplastic and neoplastic colorectal lesions [5,43], may be postulated also for adenomas among patients with the FAP syndrome but remains to be proven.

In any case, since the majority of the small adenomas are obtained from one patient, it is not possible to rule out that individual variations could have yielded these results in terms of K-ras2 mutation and aneuploidy incidences. Thus, a larger cohort of FAP patients are required to confirm our hypotheses.

In conclusion, the present data suggest that K-ras2 mutations and DNA aneuploidy are correlated to an increased cell proliferation and tumour size in a sub-

group of FAP adenomas, suggesting the possibility that these biomarkers may be used in predicting tumour progression and response to agents potentially useful to induce adenoma regression.

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