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APC gene mutations and colorectal adenomatosis in familial adenomatous polyposis

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Summary Correlations between germline *APC* mutation sites and colorectal pathophenotypes, as evaluated by the direct count of adenomas at colectomy, were investigated analysing colectomy specimens from 29 FAP patients carrying one mis-sense (codon 208) and 14 frame-shift or non-sense *APC* mutations (codons 232, 367, 437, 623, 876, 995, 1061, 1068, 1075, 1112, 1114, 1309, 1324, 1556). The mis-sense mutation at codon 208 was associated with a relatively mild colorectal pathophenotype. The mutation at codon 367, subject to alternative splicing, was associated with attenuated FAP. The mutation at codon 1309 was associated with the profuse colorectal adenomatosis. For 13 mutations, predicted to result in null alleles or truncated APC proteins, we correlated density and distribution of colorectal adenomas with the predicted functional effects of the mutation. The most severe colorectal pathophenotype was significantly associated with the truncating mutation at codon 1309, which is located downstream to the I β-catenin binding domain but upstream II β-catenin-binding domain. Mutations between codons 867 and 1114, which affect the I β-catenin binding domain, as well as mutations occurring in exons 6 and 9, predicted to result in null alleles, were associated with a less severe colorectal pathophenotype. Overall, the highest number of adenomas was detected in the right colon, followed by the left colon, transverse colon sigma and rectum. However, the highest density of adenomas was observed in the left colon, followed by the right colon, sigma, transverse colon and rectum. Colorectal carcinomas, observed in only five patients, were all in the left colon. © 2000 Cancer Research Campaign

Keywords: FAP (familial adenomatous polyposis); *APC* (adenomatous polyposis coli) gene; germline mutations; colorectal adenomas; number; distribution

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease due to mutations in the tumour suppressor adenomatous polyposis coli (*APC*) gene (Bodmer et al, 1987; Groden et al, 1991; Kinzler et al, 1991). FAP is characterized by the development of multiple adenomatous polyps throughout the large intestine (Haggitt et al, 1986; Utsunomiya, 1989) and is associated with a very high risk of colorectal cancer and with a variety of extra-colorectal disease manifestations (Jones et al, 1986; Jagelman, 1987; Baker et al, 1988; Morton et al, 1992; Olschwang et al, 1993; Caspari et al, 1995; Valanzano et al, 1996). The *APC* gene is comprised of 15 exons, of which exon 15 encodes for more than 50% of the protein (Groden et al, 1991; Kinzler et al, 1991). The APC protein is composed of 2843 amino acids and mediates growth regulatory signals by its association with the microtubule cytoskeleton and with the cadherin-binding protein β-catenin. Amino-terminal residues 1–171 are implicated in homodimerization, being the first 55 amino acids sufficient to form a stable dimer (Joslyn et al, 1993). Amino-terminal residues 453–767 contain seven copies of a repeating 42 amino acids motif, originally identified in the *Drosophila* segment polarity gene product armadillo, a component of the wingless transduction pathway (Peifer et al, 1994). Furthermore, the APC protein binds β-catenin through two motifs: the first, located between residues 1020–1169, which contains three 15 amino acid repeats (Rubinfeld et al, 1993; Su et al, 1993); the second, located between residues 1342–2075, which comprises 7 repeats of 20 amino acids (Rubilfeld et al, 1997). This second motif acts also as substrate for serine-threonine glycogen synthase kinase-3β (GSK-3β) association and phosphorylation (Rubinfeld et al, 1996). The large body of data collected on *APC* mutations in FAP has contributed to establish correlations between type and location of the mutation and clinicopathologic features of the disease. There is a wide variability in the clinical presentation of FAP, in terms of age at disease onset and number and distribution of colorectal adenomatous polyps (Giardiello et al, 1994; Presciuttini et al, 1994). Certain phenotypic characteristics of FAP appear to be correlated with the site of *APC* mutations (Spirio et al, 1993; Caspari et al, 1994; Gayther et al, 1994; Friedl et al, 1996; Soravia et al, 1998). Mutations at the very 5′ end (codons 1–157) and towards the 3′ end of the gene (i.e. beyond codon 1578) are associated with a form of FAP characterized by relatively low numbers of colorectal adenomatous polyps (5–100) and comparatively late disease onset, designated attenuated adenomatous polyposis coli (AAPC) (Leppert et al, 1990; Friedl et al, 1996; Soravia et al, 1998). Furthermore, we and others described attenuated forms of FAP in kindreds with germline mutations in regions of the *APC* gene that are subjected to alternative splicing (Curia et al, 1998). On the contrary, the most frequent *APC* germline mutation, localized at codon 1309, is associated with a very severe form of FAP, characterized by hundreds to thousands colorectal adenomas and early onset (Caspari et al, 1994; Gayther

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et al, 1994; Cama et al, 1995). Untreated patients with the codon 1309 *APC* gene mutation die on the average 10 years earlier than FAP patients with germline mutations at sites other than codon 1309 (Caspari et al, 1994; Gayther et al, 1994). Interestingly, codon 1309 lies within the 'mutation cluster region' (codons 1286–1514), i.e. the region of the *APC* gene that exhibits a definite accumulation of somatic mutations in both FAP-associated and non-FAP-associated colorectal tumours (Miyoshi et al, 1992; Miyaki et al, 1994; Yashima et al, 1994).

In spite of a number of important studies that documented the genetic bases of phenotypic heterogeneity in FAP (Leppert et al, 1990; Morton et al, 1992; Caspari et al, 1994; Gayther et al, 1994; Giardiello et al, 1994; Friedl et al, 1996; Soravia et al, 1998), there is a scarcity of data concerning correlations between number of colorectal adenomas, as evaluated by direct count on colectomy specimens, and position of the germline *APC* gene mutation responsible for the disease. The present study correlates colorectal disease manifestations with the results of *APC* mutational analysis in 29 colectomized FAP patients from 21 unrelated kindreds.

MATERIALS AND METHODS

Patients

Twenty-nine FAP patients from 21 unrelated kindreds were enroled in the study. All patients were treated with colectomy. The age at colectomy depended solely on the extent of the disease progression and ranged from 10 to 62 years (average 24.7). For each case, the area of mucosal surface of each colorectal segment (right, transverse and left colon, sigma and rectum), as well as the total area of colorectal mucosal surface, were obtained based on measurements taken on fresh colectomy specimens. The number of adenomas in the right, transverse and left colon, sigma and rectum was determined after the careful count of polyps visible on the mucosal surface of the fresh colectomy specimen and randomly confirmed by microscopic examinations. The density of adenomas per 4 cm^2 unit area of mucosal surface was calculated by dividing the number of adenomas determined in each anatomical segment, or in the entire colorectum, by the respective area of mucosal surface, expressed in cm^2. In kindred GD-11, because of the profuse adenomatosis, the number of adenomas was extrapolated by counts limited to representative 4 cm^2 unit areas of mucosal surface for each anatomical segment of the colorectum.

Mutational analysis of the APC gene

Mutational analysis of the *APC* gene was conducted after full informed consent, using genomic DNA and/or total RNA extracted from fresh peripheral blood lymphocytes. Genetic analyses were always confirmed using DNA deriving from two independent blood samples and were performed employing a combination of three screening strategies, followed by direct sequencing (Cama et al, 1991). Heteroduplex analysis on agarose minigel (HAAM), coupled with allele-specific multiplex PCR, was designed to rapidly identify the three frequently occurring deletions of the *APC* gene at codons 1061, 1068 and 1309 (Cama et al, 1995). Single-strand conformation polymorphism (SSCP) analysis was used to screen the coding sequence of the *APC* gene spanning exons 1 through 14 (Groden et al, 1991, 1993; Cama et al, 1994). The in vitro synthesized protein (IVSP) assay was used for the rapid identification of truncating mutations in exon 15 (van der Luijt et al, 1994; Valanzano et al, 1996). Primers used for amplifications were as reported elsewhere (Groden et al, 1991, 1993; Miyoshi et al, 1992; van der Luijt et al, 1994). The research protocol was approved by the ethical review board of the University Gabriele D'Annunzio.

Statistical analysis

The total number of adenomas at colectomy, the density of adenomas per unit area of mucosal surface obtained for the entire colorectum and for each colorectal anatomical segment were correlated with age at colectomy and with site of *APC* mutation. Statistical evaluations were performed using the analysis of variance (ANOVA-test). Probability values (*P*-values) of less than 0.05 were considered significant.

RESULTS

The combined use of the HAAM, SSCP and IVSP assays for mutational analysis allowed the detection of 15 different germline *APC* mutations responsible for disease in 29 FAP patients from 21 unrelated kindreds (Table 1 and Figure 1). Ten mutations were clustered at the 5′ end of exon 15, between codons 876 and 1556. Five mutations were respectively located within exons 5 (codon 208), 6 (codon 232), 9 (codons 367 and 437), and 14 (codon 623). With the exception of the novel mis-sense mutation at codon 208, all other mutations are predicted to introduce premature termination signals. The mutation at codon 1324 determines a frame-shift introducing a distant stop signal 90 codons downstream. The mis-sense mutation at codon 208 is considered pathogenic on the basis of its co-segregation with 3 FAP patients in the kindred (information kindly supplied by Dr L Varesco, Istituto Nazionale Ricerca Cancro, Genova, Italy and confirmed in our laboratory) and lack of segregation in unaffected members of the family. This APC allelic variant is unlikely to be a common polymorphism as it has not been detected in additional 118 chromosomes screened.

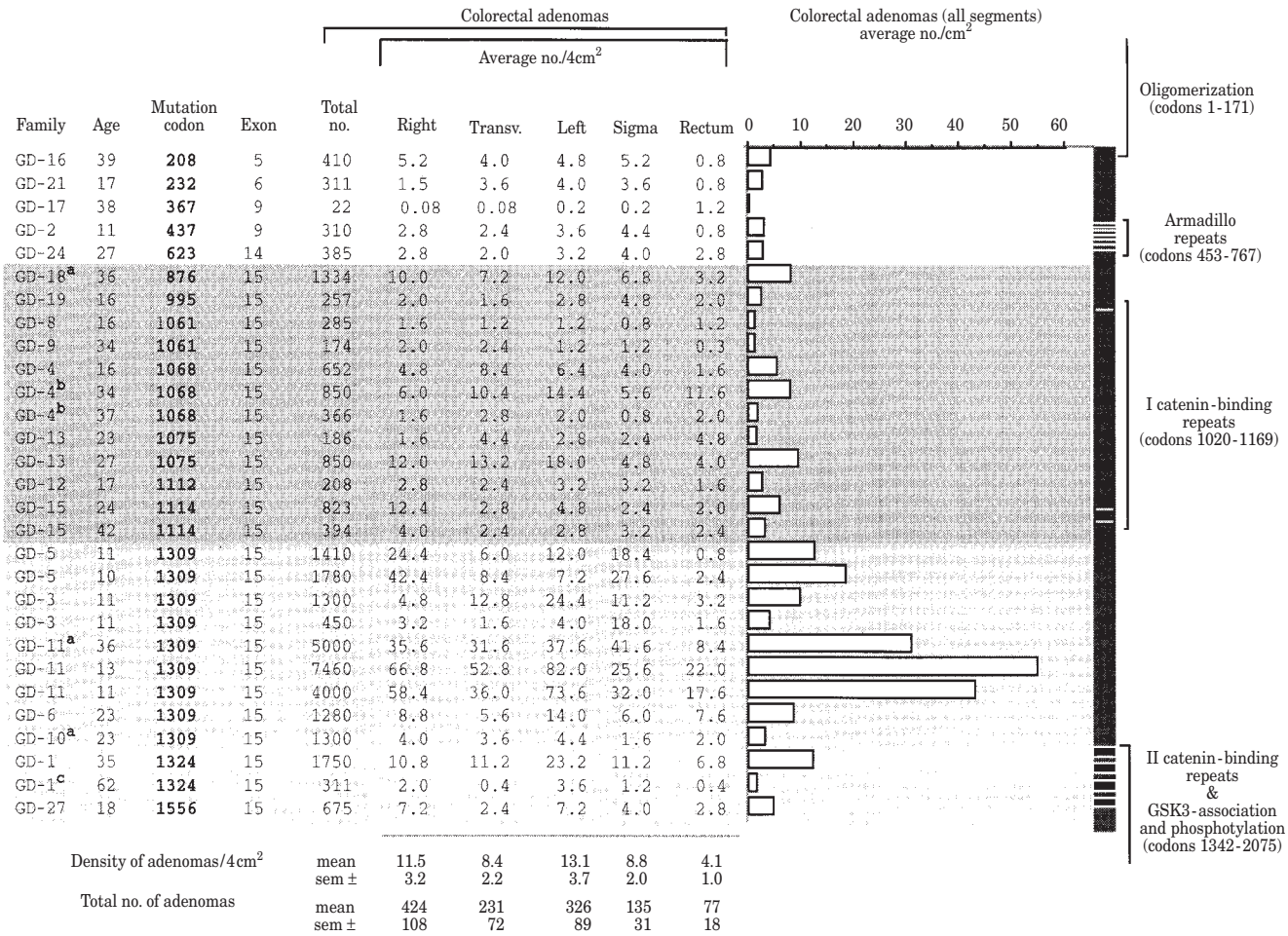
Clinicopathological parameters, including age at colectomy, total number and anatomical distribution of colorectal adenomas, density of adenomas per unit area of mucosal surface were correlated with *APC* mutation sites. The total number of colorectal adenomas detected on fresh at colectomy specimens appeared to vary among FAP patients within the same family, as exemplified in kindreds GD-1, GD-3, GD-4, and GD-15 (Figure 1). These differences appeared to be independent from age at colectomy. Variability in the total number of adenomas was also observed among FAP patients from unrelated families carrying the same *APC* mutation (Figure 1). In spite of this variability, carriers of the codon 1309 mutation manifested a significantly higher number of adenomas at colectomy (from 450 to 7460), compared to carriers of other APC mutations (from 22 to 1750) ($P < 0.01$). There was a significant anticipation in the age at colectomy in carriers of mutation at codon 1309 (average 16.6; s.e.m. ± 2.9) versus carriers of other *APC* mutations (average 28.5; s.e.m. ± 2.8) ($P < 0.01$). Six out of the ten codon 1309 mutation carriers underwent colectomy during the 2nd decade of life (Figure 1).

Colorectal cancers occurred in five patients, respectively carrying mutations at codon 876 (one patient), 1068 (two patients) and 1309 (two patients) (Figure 1). It should be noted that only one patient had cancer before age 30 years and that this patient carried

Table 1 Summary of the germline mutations of the APC gene detected in 21 FAP kindreds

Kindred	Mutation	Codon	Type of mutation	Exon
GD-16	CCA → CCG	208	Gln → Arg	5
GD-21	CGA → TGA	232	Arg → Stop	6
GD-17	GAC TCT → GAC T	367	2 bp deletion	9
GD-2	AAT CC*A A/g*tatg → AAT CC /tatg	437	3 bp deletion	9 (donor site)
GD-24	TAC CGG → TAC TTAC CGG	623	4 bp insertion	14
GD-18	CGA → TGA	876	Arg → Stop	15
GD-19	TGC → TGA	995	Cys → Stop	15
GD-8	AA ATA AAA CAA AGT → AA AT AA AGT	1061	5 bp deletion	15
GD-9	AA ATA AAA CAA AGT → AA AT AA AGT	1061	5 bp deletion	15
GD-4	AGA CAA TCA AGG A → AGA CA AGG A	1068	4 bp deletion	15
GD-13	TAT → TAA	1075	Tyr → Stop	15
GD-12	GAA *A*CA AAT → GAA CA AAT	1112	1 bp deletion	15
GD-15	CGA → TGA	1114	Arg → Stop	15
GD-3	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-5	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-6	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-7	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-10	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-11	AAA GAA AAG ATT → AAA GA TT	1309	5 bp deletion	15
GD-1	CCA GCA → CA GCA	1324	1 bp deletion	15
GD-27	GCA GAA → GCA A GAA	1556	1 bp insertion	15

HAAM, coupled with allele-specific multiplex PCR allowed to detect mutations at codons 1061, 1068 and 1309. SSCP allowed to detect mutations at codons 208, 232, 367, 437, 623, 1075 and 1324 and IVSP allowed to detect mutations at codons 876, 995, 1112, 1114 and 1556.



Family	Age	Mutation codon	Exon	Total no.	Right	Transv.	Left	Sigma	Rectum
GD-16	39	**208**	5	410	5.2	4.0	4.8	5.2	0.8
GD-21	17	**232**	6	311	1.5	3.6	4.0	3.6	0.8
GD-17	38	**367**	9	22	0.08	0.08	0.2	0.2	1.2
GD-2	11	**437**	9	310	2.8	2.4	3.6	4.4	0.8
GD-24	27	**623**	14	385	2.8	2.0	3.2	4.0	2.8
GD-18[a]	36	**876**	15	1334	10.0	7.2	12.0	6.8	3.2
GD-19	16	**995**	15	257	2.0	1.6	2.8	4.8	2.0
GD-8	16	**1061**	15	285	1.6	1.2	1.2	0.8	1.2
GD-9	34	**1061**	15	174	2.0	2.4	1.2	1.2	0.3
GD-4	16	**1068**	15	652	4.8	8.4	6.4	4.0	1.6
GD-4[b]	34	**1068**	15	850	6.0	10.4	14.4	5.6	11.6
GD-4[b]	37	**1068**	15	366	1.6	2.8	2.0	0.8	2.0
GD-13	23	**1075**	15	186	1.6	4.4	2.8	2.4	4.8
GD-13	27	**1075**	15	850	12.0	13.2	18.0	4.8	4.0
GD-12	17	**1112**	15	208	2.8	2.4	3.2	3.2	1.6
GD-15	24	**1114**	15	823	12.4	2.8	4.8	2.4	2.0
GD-15	42	**1114**	15	394	4.0	2.4	2.8	3.2	2.4
GD-5	11	**1309**	15	1410	24.4	6.0	12.0	18.4	0.8
GD-5	10	**1309**	15	1780	42.4	8.4	7.2	27.6	2.4
GD-3	11	**1309**	15	1300	4.8	12.8	24.4	11.2	3.2
GD-3	11	**1309**	15	450	3.2	1.6	4.0	18.0	1.6
GD-11[a]	36	**1309**	15	5000	35.6	31.6	37.6	41.6	8.4
GD-11	13	**1309**	15	7460	66.8	52.8	82.0	25.6	22.0
GD-11	11	**1309**	15	4000	58.4	36.0	73.6	32.0	17.6
GD-6	23	**1309**	15	1280	8.8	5.6	14.0	6.0	7.6
GD-10[a]	23	**1309**	15	1300	4.0	3.6	4.4	1.6	2.0
GD-1	35	**1324**	15	1750	10.8	11.2	23.2	11.2	6.8
GD-1[c]	62	**1324**	15	311	2.0	0.4	3.6	1.2	0.4
GD-27	18	**1556**	15	675	7.2	2.4	7.2	4.0	2.8
Density of adenomas/4cm²				mean	11.5	8.4	13.1	8.8	4.1
				sem ±	3.2	2.2	3.7	2.0	1.0
Total no. of adenomas				mean	424	231	326	135	77
				sem ±	108	72	89	31	18

Figure 1 Age at colectomy, site and exon of *APC* gene mutations, total number of colorectal adenomas and average number of adenomas per unit area of mucosal surface in the colorectal anatomical segments. The histogram illustrates the average number of adenomas per unit area of colorectal mucosa, as evaluated for the entire colorectum in each patient. A schematic representation of the APC functional domains affected by the mutations is shown to the right (not in scale). (a), patients affected by invasive cancer; (b), patients affected by 'in situ' colorectal cancer; (c) patients affected by duodenal cancer at 62 years of age

the codon 1309 mutation. A carrier of the codon 1324 mutation, affected with duodenal carcinoma, was still negative for colorectal cancer in the seventh decade.

Considering all the 29 patients and regardless of the relative areas of mucosal surface of each colonic segment, the highest number of adenomas was localized in the right colon, followed by the left and transverse colon, sigma and rectum (Figure 1). However, the density of adenomas per unit area of mucosal surface (4 cm^2) was highest in the left colon, followed by the right colon, sigma, transverse and rectum (Figure 1).

To investigate possible correlations between the colorectal pathophenotypes and the predicted effects of the mutations on the APC protein, we focused on frame-shift and splice-site mutations. The novel mis-sense mutation at codon 208 and the previously described mutation at codon 367, that was associated to a transcript dosage effect resulting in an attenuated phenotype (Curia et al, 1998), were not considered for these specific correlations. The 27 FAP patients carrying truncating mutations were divided into three subsets (Figures 1 and 2). Subset no. 1 included patients with mutations at codons 232, 437 and 623, proven or predicted to result in lack of expression of the mutant alleles (Smith et al, 1993; Curia et al, 1998). Subset no. 2 carried mutations at codons 876, 995, 1061, 1068, 1075, 1112 and 1114, predicted to result in APC proteins truncated downstream to the armadillo repeats but upstream to or within the I β-catenin-binding repeats. Subset no. 3 carried mutations at codons 1309, 1324 and 1556, predicted to result in APC proteins truncated downstream to the I β-catenin-binding repeats but upstream to or within the II β-catenin-binding repeats and GSK-3β association and phosphorylation sites. The three subsets of patients demonstrated differences in the mean number of total colorectal adenomas, that corresponded to 335 (s.e.m. ± 24) for subset no. 1, to 531 (s.e.m. ± 106) for subset no. 2, and to 2226 (s.e.m. ± 623) for subset no. 3 (P = 0.009), as well as in the average number of adenomas per units of area of colorectal mucosa (Figure 2). Subset no. 2 tended to associate with higher number and density of adenomas than subset no. 1, but the differences between these subsets were not statistical. The most severe colorectal pathophenotype, in terms of total number and density of adenomas per unit area of mucosal surface, was associated with subset no. 3 (Figure 2).

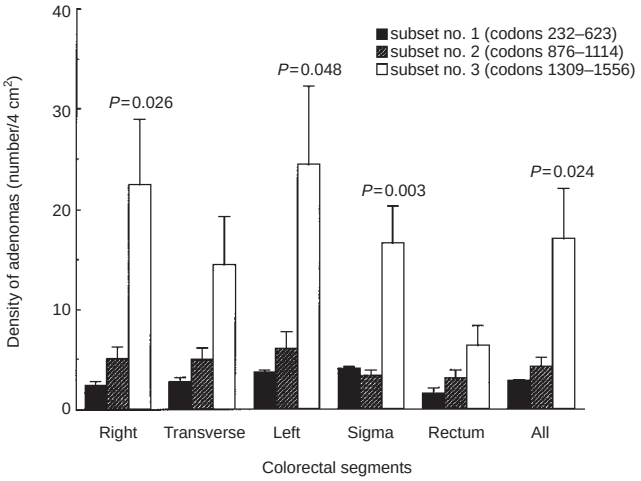


Figure 2 Average number of colorectal adenomas in the three subsets of FAP patients with *APC* mutations predicted to result in truncated proteins or null alleles. Mutations at codons 208 and 367 are not considered in this histogram. Probability values (P) calculated by analysis of variance (ANOVA-test)

DISCUSSION

The vast majority of FAP patients in our series carried mutations in exon 15 and the most frequent was the 5-bp deletion at codon 1309, which is in agreement with other studies (Nagase et al, 1992; Gayther et al, 1994). Ten of the 15 *APC* mutations discussed in the present study are predicted to result in the expression of proteins truncated at various levels in the carboxyterminal domain, that contains important growth suppressor activities (Nagase et al, 1993; Smith et al, 1993; Munemitsu et al, 1994; Matsumine et al, 1996). Four of the 15 mutations occurring between codons 232–623 are predicted to result in null alleles (Smith et al, 1993; Curia et al, 1998). Among these, the mutation at codon 367 in exon 9 is attenuated because of differential splicing (Curia et al, 1998) and was associated with a colorectal pathophenotype within the clinicopathological spectrum of AAPC (Leppert et al, 1990; Soravia et al, 1998). Finally, one of the 15 mutations resulted in the replacement of a negatively-charged with a positively-charged amino acid residue at codon 208. The functional implications of this mutation are unclear, although the mis-sense variant was associated with a relatively mild colorectal pathophenotype.

A crucial function of the multidomain APC protein is that of cooperating with GSK-3β in the regulation of free β-catenin level (Dietrich et al, 1997; Morin et al, 1997; Sparks et al, 1998). The association of β-catenin with its binding sites in the APC protein and the phosphorylation of APC by GSK-3β are prerequisites for β-catenin down-regulation. Free cytoplasmic β-catenin and Tcf family proteins are implicated in the constitutive activation of a signalling pathway promoting cell growth and differentiation, which is suspected to cause adenoma formation following *APC* gene mutation (Dietrich et al, 1997; Morin et al, 1997; Sparks et al, 1998). For 13 mutations predicted to result in null alleles or truncated APC proteins we correlated number and distribution of colorectal adenomas with the predicted functional effects of the *APC* mutations. In our series, the density of colorectal adenomas was not statistically different between mutations predicted to result in a null allele and mutations predicted to truncate the protein before or within the I β-catenin binding repeats. Conversely, mutations predicted to result in APC proteins truncated downstream to the I β-catenin-binding site but upstream to or within the II β-catenin-binding repeats and GSK-3β association and phosphorylation sites were associated with a more aggressive phenotype in terms of density of colorectal adenomas. As in other studies (Caspari et al, 1994; Gayther et al, 1994), the most aggressive colorectal pathophenotype, both in terms of overall number of colorectal adenomas and of age at colectomy, was associated with the truncating mutation at codon 1309, which is located downstream to the I β-catenin-binding repeats, but upstream to the II β-catenin-binding repeats, located between codons 1342 and 2075 (Rubinfeld et al, 1997). Intriguingly, excluding patients with codon 1309 mutation, the highest density of adenomas was observed in one patient with a mutation at codon 1324, that is localized just before the II β-catenin-binding repeats. The possibility that also other mutations localized in this region may associate with a more aggressive phenotype should be further

investigated. In support of this possibility, a previous study showed that mutations localized between codons 1250–1330 (i.e. between the two β-catenin-binding repeats), are associated with a 'profuse' type of polyposis (Nagase et al, 1993).

Regardless of mutation site, the left colon consistently harboured the highest density of adenomas per unit area of mucosal surface, followed by the right colon, sigma, transverse colon and rectum. Regardless of the relative areas of mucosal surface of each colonic segment, the predominance of the absolute number of adenomas in the right colon might account for the prevalently right-sided manifestations of AAPC (Lynch and Smyrk, 1998; Soravia et al, 1998). The degree of phenotypic heterogeneity among patients carrying the same *APC* mutation supported the view that *APC* modifying genes and/or epigenetic and environmental factors may influence the expression of colorectal disease (Borenstein and Dove, 1993; Giardiello et al, 1994; Moser et al, 1995). The colorectal carcinomas, observed in five patients, were all in the left colon, suggesting that regionally prevalent cancer promoting factors might favour left sided adenoma–carcinoma transition.

In conclusion, *APC* mutation sites, although not completely accounting for the intra- and inter-familial variation in the pathophenotype of FAP, provide information that may be relevant for the clinical management of colorectal adenomatosis in FAP carriers. In perspective, surveillance strategies and the timing and extent of prophylactic surgery could be influenced by the specificities of disease manifestations associated with individual APC gene mutations.

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