Frequency of the APC protein deficiency and its evaluation with regard to the malignant potential of sporadic colorectal adenomas

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Abstract. *Mutations of the APC gene have been thought to be early events in the process of colorectal carcinogenesis. Some reports, however, described different results as to the frequency of APC mutations between adenomas and carcinomas. The aim of our study was to evaluate the frequency of the loss of the APC immunoreactivity and to compare APC status with malignant potential of adenomas. We performed an immunohistochemical analysis of the APC protein in 118 adenomas and compared these results with the size, histology and grade of dysplasia as parameters of the malignant potential of colorectal adenomas. We also compared the APC immunoreactivity with the location of adenomas, type of growth, and age of patients, respectively. In addition, we assessed the APC status of colorectal cancer tissues from 9 patients. We found complete loss of APC protein in 28 (23.7 %) adenomas, sixty-three (53.4 %) were unequivocally positive, and remaining 27 cases (22.9 %) showed partial positivity. Among carcinomas, seven tissues (77.8 %) were negative as to the APC immunoreactivity. Using the Kruskal-Wallis ANOVA test to compare the APC status with the size of adenomas and age of patients, and M-L chi-square test to assess the relationship between the APC status and histology, degree of dysplasia, location, and age of patients, we did not find any correlation between the type of APC immunostaining and parameters assessed. As the APC immunoreactivity differs substantially between adenomas and carcinomas, we suggest that complete loss of APC protein should be assessed as a risk factor for malignant transformation regardless of other morphological parameters of adenomas.*

Key words: *adenoma, colorectal cancer, APC gene, immunohistochemistry*

Bortlík M, Vítková I, Papežová M, Kohoutová M, Novotný A, Adamec S, Chalupná P, Lukáš M. Deficit APC proteinu a jeho vztah k malignímu potenciálu sporadických adenomů tlustého střeva. Folia Gastroenterol Hepatol 2004; 2 (4): 174 - 182.

Souhrn. *Mutace APC genu jsou považovány za časnou událost v procesu onkogeneze kolorektálního karcinomu. Řada prací však prokázala rozdíly ve výskytu mutace APC mezi adenomy a karcinomy. Cílem naší studie bylo zhodnotit frekvenci ztráty APC imunoreaktivity ve tkáni kolorektálních adenomů a porovnat výskyt APC proteinu s parametry maligního potenciálu adenomů. Za tímto účelem bylo provedeno imunohistochemické vyšetření APC proteinu ve 118 adenomech tlustého střeva a byl porovnán jeho výskyt s velikostí adenomů, histologickou strukturou a stupněm dysplázie jakožto parametry maligního potenciálu střevních adenomů. Dále byl porovnán APC status s lokalizací adenomu, typem růstu a věkem pacienta. Paralelně bylo imunohistochemicky vyšetřeno 9 vzorků tkáně kolorektálního karcinomu. Ve skupině adenomů byla zjištěna kompletní absence APC proteinu ve 28 případech (23,7 %), 63 adenomů (53,4 %) mělo imunoreakci pozitivní, zbylých 27 (22,9 %) ukázalo částečnou pozitivitu. Ve skupině karcinomů jsme kompletní ztrátu APC proteinu zjistili v 7 případech (77,8 %). Statistická analýza provedená pomocí Kruskal-Wallisova ANOVA testu (APC status vs. velikost adenomu a věk pacientů) a M-L chi-kvadrát testem (APC vs. histologický typ, stupeň dysplazie, lokalizace adenomu a typ jeho růstu) neprokázala žádnou souvislost mezi výsledkem imunoreakce APC proteinu a některým z parametrů maligního potenciálu, resp. ostatními sledovanými parametry. Vzhledem k výraznému rozdílu APC pozitivity mezi adenomy a karcinomy však považujeme ztrátu APC imunoreaktivity za rizikový faktor maligní transformace adenomu bez ohledu na další morfologické parametry.*

Klíčová slova: *adenom, kolorektální karcinom, APC gen, imunohistochemie*

Introduction

Carcinogenesis of the colorectal cancer includes a rank of genetic events underlying a sequential conversion of the normal colonic mucosa into the malignant tumour. The adenomatous polyposis coli (APC) gene is thought to play a key role in the pathogenesis of colorectal cancer not only in patients with familial adenomatous polyposis syndrome, but also in the majority of patients with sporadic colorectal neoplasia (5). About 60 - 80 % of sporadic carcinomas harbour a mutation in the APC gene and similar frequency was reported in colorectal adenomas (11,12,15). Mutation in the APC gene is thus supposed to be an early event in the process of colorectal carcinogenesis (13).

APC gene implicated in the process of the neoplastic conversion leads to a stop codon with subsequent synthesis of a truncated APC protein that lost the carboxyl-terminal part (4,13). Loss of this part of the APC protein results in the loss of almost all binding sites for beta-catenin, which, if not bound in a multiprotein complex with APC, translocates into the nucleus, where it promotes the process of malignant transformation (4). The absence of the full-length and functional APC protein can be detected by the immunohistochemistry using rabbit or mouse polyclonal antibodies. When compared with other gene-analysis techniques, immunohistochemistry is cheaper, easily performed and a less time-consuming procedure.

Iwamoto et al. (6) reported on the results of an immunohistochemical analysis where the frequency of the complete loss of the APC protein was substantially lower in colorectal adenomas as compared with carcinomas. Other authors also studied the relationship between the APC protein expression and morphological features of colorectal neoplasms, but results are still conflicting (7,9,10). Therefore, we performed our study to evaluate the relationship between the loss of the full-length APC protein assessed by immunohistochemistry and morphological criteria of the malignant potential of the adenoma. In addition, correlation between the type of APC immunoreactivity and the location of the polyp, its type of growth (sessile vs. pedunculated) and age of patient were assessed. We compared our results obtained in adenomas with findings found in a small group of patients with carcinomas.

Patients and methods

Between January 2003 and August 2003 we performed 843 colonoscopic examinations; of them, one hundred and eighteen patients (71 male, 47 female, age 25 - 86 years, mean 63 years) with endoscopically removed colorectal adenomas were included in this study. Patients with known diagnosis of the familial adenomatous polyposis were not included. In total, 182 adenomas were endoscopically removed, between 1 - 10 adenomas per patient (median 1). In patients with more than one adenoma, only the largest one

was used for further assessment as described below. In summary, 118 adenomas were examined.

In all patients, polyps were removed either by the technique of snare polypectomy or, when smaller than 5 mm, by classical biopsy forceps. Sessile adenomas above 10 mm of size were usually removed by the technique of the endoscopic mucosal resection (2). Eighty-three adenomas (70.3 %) were located in the left side of the large bowel (rectum, sigmoid colon, descending colon), remaining 35 (29.7 %) were found in the right colon. Only 31 adenomas (26.6 %) were pedunculated polyps, eighty (67.8 %) were broadly based polyps and 8 (6.8 %) adenomas had sessile, non-polypoid appearance. Among 118 tumours, 82 (69.5 %) were tubular, 32 (27.1 %) tubulo-villous, and 4 (3.4 %) villous adenomas, respectively. Low-grade dysplasia was found in 105 cases (89 %) and high-grade dysplasia in remaining 13 adenomas (11 %).

The size of each adenoma was measured either using the biopsy forceps opened close to the adenoma in the bowel or after withdrawal of the polyp from the colon. In the large adenomas resected by a peace-meal technique, the size was assessed before the adenoma was removed from the bowel. Mean size of adenomas was 14 mm (3 - 55 mm), median 6 mm, 29 adenomas (24.6 %) were 20 mm of size or larger. Immunohistochemical examination of the APC protein was then performed in all 118 adenomas. In addition, immunohistochemistry was done also in the tissue samples of nine colorectal carcinomas from patients who underwent a surgical resection.

Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the General Teaching Hospital.

Technique of the immunohistochemical analysis of the APC protein

Paraffin embedded tissue blocks were cut to obtain sections 4 μm thick, deparaffinized in xylen and rehydrated in a series of graded alcohol + water solutions. Endogenous peroxidase activity was then blocked by 3 % solution of hydrogen peroxide in methanol. Subsequently, tissues were incubated in non-immune serum for 30 min. Following this, incubation with antiAPC antibody (C-terminus, Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:200 for 60 min. was performed. Streptavidin peroxidase (detection kit) with diaminobenzidine as a substrate was used for detection under room temperature conditions. Sections were stained with haematoxylin, dehydrated, and assembled into the xylen soluble medium. A definite APC immunoreactivity in the adjacent normal mucosa of each tumour (adenoma or carcinoma) served as a control of the normal APC protein reaction.

Statistics

To assess relationships between continual and categorial variables, the Kruskal-Wallis ANOVA test after Kolmogorov-Smirnov analysis was used. The Kruskall-Wallis ANOVA was used to test the relationship between APC status and the size of adenomas and between APC status and age of patients. Categorial data were analyzed using the M-L (maximum likelihood) chi-square test. This analysis was performed to test the relationship between the APC status and histology, degree of dysplasia, location, and type of growth, respectively. All tests were bi-lateral and the significance level was set at 5 % for each analysis.

Results

Immunohistochemical analysis revealed 4 types of staining:

Group 1: Definite APC positivity (Figure 1). This result means diffuse APC immunoreactivity, characterized by the brown staining present in the cytoplasm of epithelial cells. Totally, 63 adenomas (53.4 %) were APC positive.

Group 2: Definite APC negativity (Figure 2) was found in 28 adenomas (23.7 %). No staining in any part of an adenoma despite the positivity in the adjacent normal mucosa resulted in this assessment.

Group 3: Focal APC positivity (Figure 3) was described in 16 adenomas (13.6 %). Immunostaining was disperse, but not diffuse throughout the adenomatous tissue.

Group 4: Peripheral positivity of APC protein, found in 11 adenomas (9.3 %). In these cases, the immunostaining was present only at the peripheral part of the adenoma adjacent to the normal, non-tumorous mucosa.

Immunohistochemical evaluation of the tissue from 9 patients with colorectal cancer showed lack of an APC staining in 7 cases (77.8 %), another 2 patients displayed focal positivity.

1. Relationship between APC immunoreactivity and malignant potential of adenomas

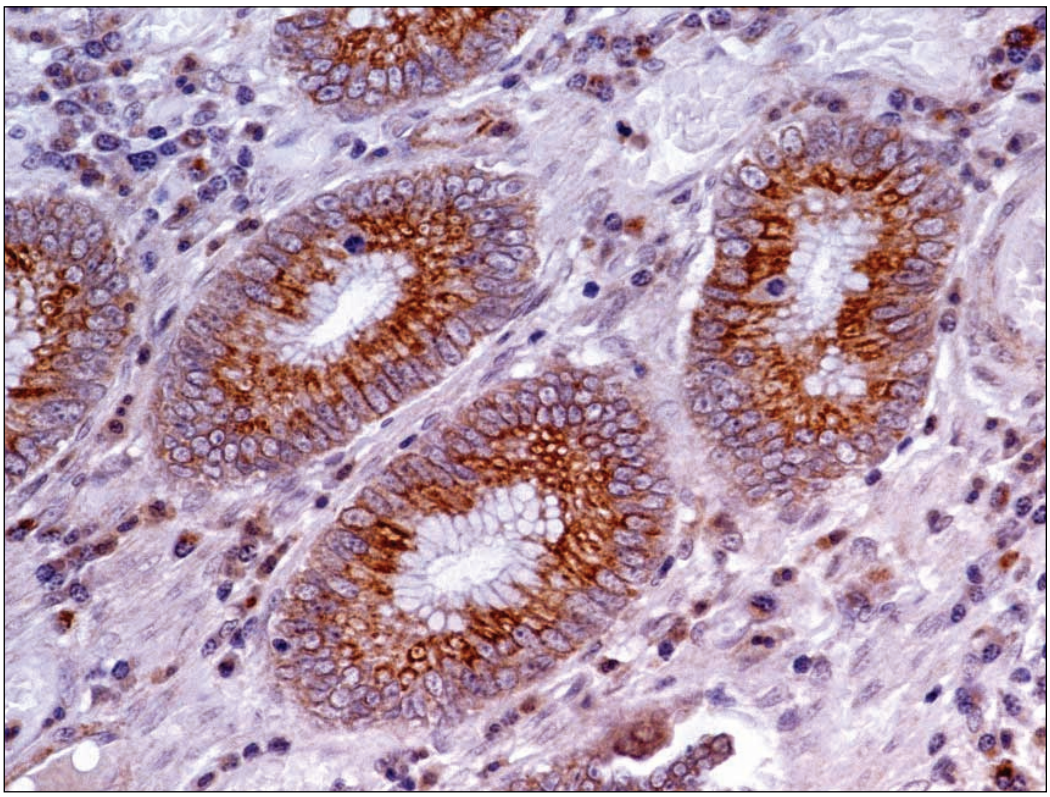

Figure 1
Diffuse positivity (brown colour) in the cytoplasma of epithelial cells of the adenoma - presence of the full-length APC protein. Immunohistochemical staining.

Difúzní pozitivita (hnědé zbarvení) v cytoplazmě epiteliálních buněk adenomu - přítomnost APC proteinu. Imunohistochemie.

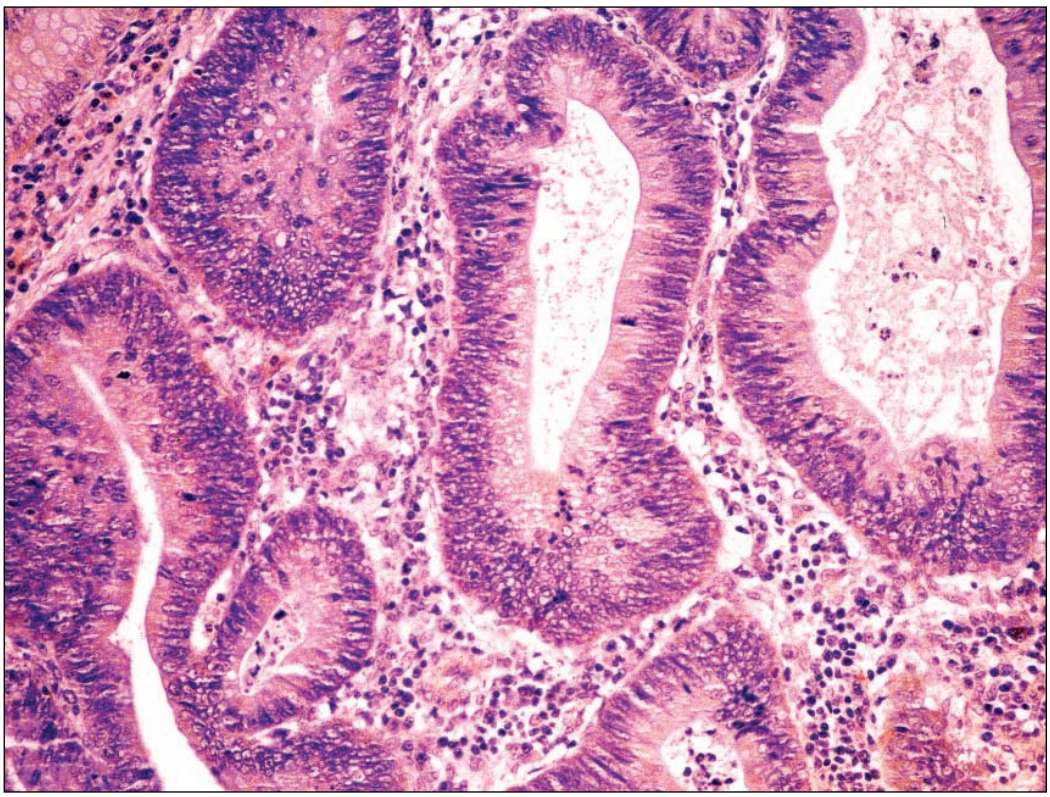

Figure 2
Absence of immunoreactivity due to the complete loss of the full-length APC protein. Immunohistochemical staining.

Chybějící imunoreaktivita v důsledku úplné ztráty APC proteinu. Imunohistochemie.

The malignant potential of the adenomatous polyps is determined by the size of an adenoma, degree of dysplasia and histological characteristic, namely the presence of the villous structures. Results of this assessment are shown in Table 1.

a) Size of adenoma and the APC protein expression

When all four types of immunostaining (positive, negative, focal and peripheral) were considered and compared with the size of adenomas, no statistically important difference was found between any group evaluated ($p = 0.299$). Since the polyps above 20 mm of size are considered as large, with an increased risk

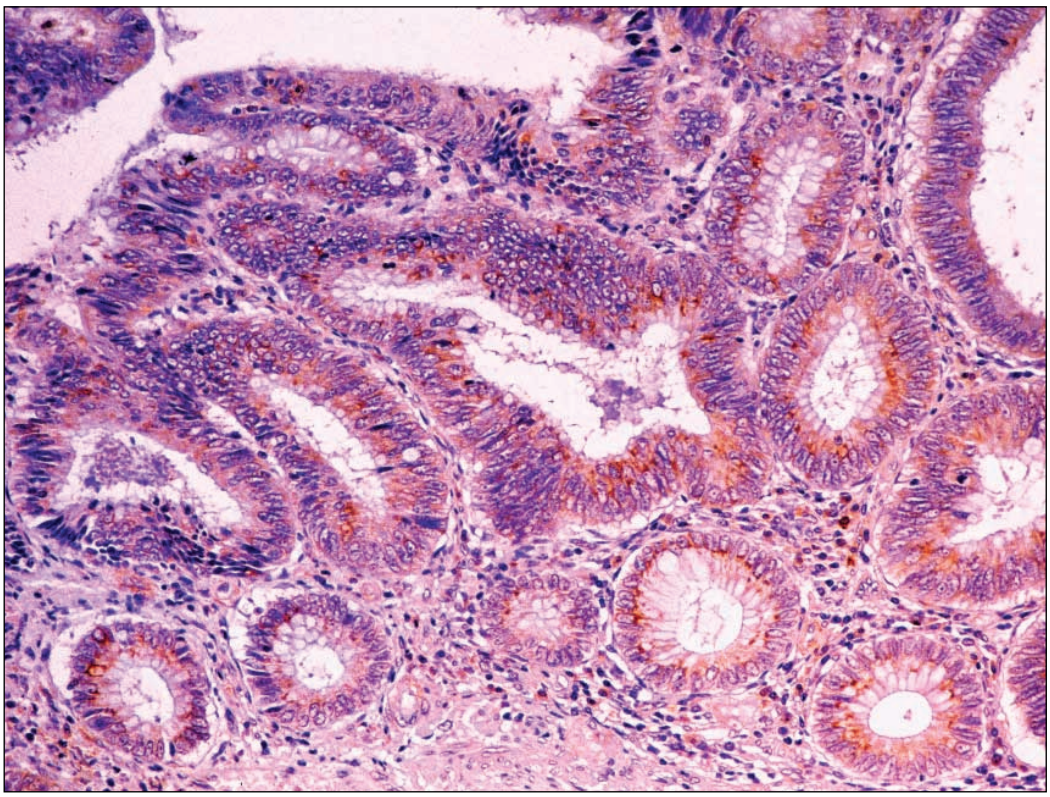

Figure 3
Focal positivity of the immunoreaction for APC protein. Immunohistochemical staining.

Fokální pozitivita imunoreakce na APC protein. Imunohistochemie.

of malignant transformation, we divided our group according to this criterion into the adenomas below 20 mm and those reaching 20 mm of size or larger. Only adenomas with either definite APC positivity or definite APC negativity entered this additional evaluation. The frequency of APC negative adenomas among smaller polyps (below 20 mm) was 30 % (20 out of 67) and did not significantly differ from the proportion of the APC negative large adenomas (33 %, 8/24).

b) Histology and the APC protein
Adenomas were assessed according to their histological feature (tubular, tubulo-villous and villous), and compared with the type of the APC immunoreaction. Again, we did not observed any significant relationship between the histology and APC status (p = 0.239). Similar proportion of all APC immunoreactions among histological subgroups was found even when the number of pure villous adenomas included was relatively small (only 4).

c) Grade of dysplasia and the APC protein
To compare the APC protein expression with degree of dysplasia, we divided entire group of adenomas into those with low-grade (which included mild and moderate dysplasia) and those with high-grade dysplasia. Statistical

Table 1
Comparison of each parameter of the malignant potential (left column) with immunohistochemical assessment. The right column shows resultant p values.

Srovnání jednotlivých parametrů maligního potenciálu (levý sloupec) s imunohistochemickým vyšetřením. V pravém sloupci jsou hodnoty p.

Parametr		positive	negative	focal	peripheral	p
size (mm)	mean (SD)	13.7 (14.7)	14.5 (15.8)	10.2 (13.4)	17.9 (13.4)	0.299
	median	6	5	5	15	
histology	T	41	20	14	7	0.239
	TV	18	8	2	4	
	V	4	0	0	0	
grade of dysplasia	low-grade	54	26	15	10	0.807
	high-grade	9	2	1	1	

T - tubular, TV - tubulo-villous, V - villous

Table 2
Comparison of other parameters (left column) with immunohistochemical assessment. The right column shows resultant p values.

Srovnání ostatních parametrů (levý sloupec) s imunohistochemickým vyšetřením. V pravém sloupci jsou hodnoty p.

parameter		positive	negative	focal	peripheral	p
location	rectum	15	3	3	2	0.549
	sigmoid c.	29	11	5	5	
	descending c.	3	4	1	2	
	transverse c.	5	4	2	1	
	ascending c.	6	5	2	1	
	caecum	5	1	3	0	
type of growth	sessile	47	21	12	6	0.545
	pedunculated	16	7	4	5	
age (years)	mean (SD)	63.4 (12.6)	62.2 (16.3	61.7 (13.7)	65.2 (14.9)	0.879
	median	62	68	64	68	

analysis did not found any significant relationship between the type of APC immunoreactivity and dysplastic changes in the adenomatous tissue (p = 0.807).

In general, we were not able to find any relationship between the type of APC protein expression and parameters of the malignant potential, such as size of adenoma, histology and degree of dysplasia.

2. Relationship between the APC protein immunostaining and other parameters

Besides the malignant potential, we looked at the site, where each adenoma was found, type of its growth and age of the patient as parameters potentially being related to the type of APC protein expression. The results are shown in Table 2.

a) Polyp location and the APC protein
When all types of APC immunostaining were considered, no association with the colonic location of adenomas was appreciated (p = 0.549). Moreover, we performed a separate assessment of APC negative and APC positive adenomas divided according to their location in the right (coecum, ascending and transverse colon) or left (rectum, sigmoid and descending colon) part of the large bowel. There was a trend towards higher proportion of APC negative adenomas located proximally (39 %) as compared with APC negative distal tumours (28 %).

b) Adenoma shape (type of growth) and the APC protein expression
Type of growth (pedunculated or sessile) of each adenoma was compared with results of APC immunostaining. Again, both these groups did not differ with regard to the APC protein expression (p = 0.545).

c) Age of patient and the APC protein
Finally, no statistically significant relationship between the type of APC immunoreaction and the age of patients was detected (p = 0.879).

Discussion

We performed an analysis of the expression of the APC protein in the tissue of sporadic colorectal adenomas to assess the frequency of deficiency of this protein with regard to the malignant potential of the sporadic adenomas. Other parameters, such as location of the adenoma, its shape and age of patient were also analyzed in this study. We used an immunohistochemichal reaction as a parameter documenting the presence or absence of the full-lenght APC protein. A rabbit antibody against the peptide corresponding to the last 20 amino-acids carboxy-terminus was used for this purpose. We attempted to verify the hypothesis that the absence of the full-length APC protein resulting from bi-allelic mutation of the APC gene (or mutation of one and loss of the second allele) might be associated with a higher risk of the malignant transformation of the adenoma assessed by the parameters of the malignant potential. We found, however, a complete loss of the APC immunoreactivity in a relatively small proportion of adenomas (23.7 %), more than one half of adenomas had prominent APC immunoreactivity (53.4 %). Moreover, we detected almost one quarter of samples showing focal

or peripheral APC protein positivity (22.9 %). Comparison of any particular type of the immunoreaction (positive, negative, focal and peripheral) with parameters of the malignant potential did not confirm a statistically significant relationship between them. We have not found any relationship even between the APC immunostaining and other parameters such as location and shape of adenoma or age of the patient.

It is necessary to emphasize that immunohistochemistry detects the presence of the full-length APC protein instead of the mutation of the gene as itself. It is a current assumption that almost all of the mutations of the APC gene result in the production of a truncated protein that has lost its tumour suppressor function (4). The absence of the full-length APC protein thus indicates a bi-allelic loss of the gene caused by two mutations or by combination of mutation of one allele with another type of genetic or epigenetic event (allelic loss, promoter hypermethylation etc.). On the other hand, mono-allelic defect does not necessarily need to be followed by the loss of the APC immunoreactivity.

We have observed a substantial difference in the absence of the APC protein between adenomas and carcinomas (24 % vs. 78 %). This is, at least partially, inconsistent with the hypothesis that complete inactivation of both alleles of the APC gene occurs early during the process of colorectal tumorigenesis of most colorectal neoplasms. Thus, it is possible that a large number of adenomas contain the mutation of only one allele of the APC gene. Whereas 45 - 60 % of all adenomas and 60 - 80 % of carcinomas harbour one allele inactivated, bi-allelic defect could be found in approximately 30 % of adenomas (7,13). One possible explanation of this finding is that the carcinogenesis of the sporadic colorectal tumours differs from the carcinogenesis of tumours of patients with familial adenomatous polyposis syndrome. According to the Knudson's two-hit model, in patients with the adenomatous polyposis coli one germ-line mutation is followed by the second hit during the early period of life, and then, multiple adenomatous polyps become apparent in the large bowel. The vast majority of colorectal adenomas from patients with familial adenomatous polyposis thus harbor mutations of both alleles of the APC gene (8).

There is no clear relationship between the APC gene mutation and histology of the adenomas. While some authors found higher frequency of the APC gene mutation in tubulo-villous and villous adenomas (3), others did not (9). Although our results do not give clear information about the exact proportion of mutations in the adenomas, we have not found any relationship between the malignant potential and the absence of the full-length APC protein as an equivalent of the bi-allelic mutation. Nevertheless, this lack of significant difference between adenomas with different malignant potential is followed by an abrupt increase of the absence of the APC protein in carcinomas, which harbor a complete (bi-allelic) APC defect almost four times more frequently. Similar results were published by Iwamoto et al. (6) who found complete negativity for APC immunostaining in 83 % of carcinomas, but for only 29 % of adenomas. Moreover, even when the exact proportion of APC gene mutations or other epi-genetic changes were assessed, results were different for adenomas as compared with carcinomas (7,13). According to these results, it seems possible that, in case of sporadic colorectal adenomas, the loss of a single APC allelle is sufficient to lead to adenoma formation. Final malignant transformation then starts only when the "second hit" in the adenoma cells occurs. Sporadic adenomas may grow with only one allele inactivated thanks to, for example, dominant-negative effect of APC protein homodimers formed by a truncated protein together with the full-length APC protein (1). These homodimers prevent normal function of APC protein but not its immunoreactivity. This theory is supported by the finding of the nuclear, so that abnormal accumulation of beta-catenin in adenomas with a definite immunohistochemical positivity of the APC protein (6).

We can see a small drawback in assessment of our results that is, in our oppinion, a small number of pure villous adenomas and adenomas with a high-grade dysplasia. Nevertheless, the proportion of villous as well as highly dysplastic adenomas is similar as reported by most authors (5 - 10 % each) and statistical methods used are capable of minimizing this bias.

Our results seem to be somewhat complicated due to the fact that we observed four different types of immunoreactions. Besides the definite positivity or negativity in the APC immunostaining, there were many adenomas with either focal or peripheral positivity. This "partial" APC protein expression within adenomatous tissue was also described by others,

even when less frequently (6). It could be, for instance, due to the presence of the genotypically heterogenous tissue, where a single APC mutation present in all cells is combined with the bi-allelic inactivation in only some parts of the same adenoma. Other possible explanation is that two distinct types of clonal expansion exist in the adenoma since the beginning of its growth. As the complete absence of the APC protein gives a growth advantage to the cells, one can assume that the partial deficiency of APC protein should be a step towards its complete absence.

A key question is whether the presence or absence of the full-length APC protein affects the prognosis of adenomas as to their future malignant transformation. Theoretically, the lack of the APC protein should stimulate adenomas to grow and undergo malignant transformation. While several reports, including our results, showed no different morphology of APC negative and positive adenomas (6,9), others found that the prevalence of APC mutation continually increased from normal mucosa to carcinoma (7), or that the frequency of APC mutations in tubulo-villous or villous adenomas was higher than in tubular adenomas (10). Among our adenomas, a lack of APC protein was observed even in some polyps smaller than 5 mm, which are generally thought to be of minimal clinical importance. On the other hand, several investigators have observed that there are many other genes involved in colorectal carcinogenesis and that significant number of adenomas, as well as carcinomas, do not harbour any APC gene mutation at all (7,14). A substantial difference between the frequency of the APC negative adenomas and carcinomas may suggest that a complete absence of the full-length APC protein in adenomas increases the risk of its malignant transformation. Simultaneously, APC mutations appear to play an important role not only during the initial phase but also during the conversion of adenoma into carcinoma.

In conclusion, we have not observed an unequivocal relationship between the presence or absence of the full-length APC protein and degree of malignant potential of colorectal adenoma, its location in the bowel, type of growth, and age of patient, respectively. There was a slightly increased frequency of APC negative adenomas localized in the proximal part of the colon. Due to the high frequency of the APC negative carcinomas as compared with adenomas, it seems probable that complete lack of APC immunoreactivity in an adenoma may signalize a higher risk of malignant transformation irrespective of the current morphology. Long-term follow-up of patients after endoscopic polypectomy should answer the question, whether patients with initial APC protein negativity have a higher risk of the local recurrence or metachronous adenoma elsewhere in the large bowel.

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