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ORIGINAL PAPER

Somatic APC Inactivation Mechanisms in Sporadic Colorectal Cancer Cases in Hungary

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Abstract The role of germline inactivation of the adenomatosis polyposis coli (APC) gene in hereditary colorectal cancer is well known, being the most important cause of familial adenomatosus polyposis (FAP) syndrome. Hereditary cases with germline mutations, however, account only for 5–10% of colorectal cancers. The somatic inactivation of this gene has also been observed in sporadic cases. In order to examine the inactivation mechanisms of the APC gene we screened 70 sporadic colorectal cancer cases (27 rectal, 43 intestinal) of different stages for promoter hypermethylation, allelic imbalance (AI) and somatic mutations. The presence of promoter hypermethylation was observed in 21 cases (30%). Fifteen of the examined tumors (21%) showed AI, and also 15 tumors (21%) carried at least one somatic mutation. Thirteen of the detected alterations were novel variations: seven frameshifts, four missense mutations and two polymorphisms. Biallelic inactivation was found in 15 patients (21%). These results suggest that the inactivation of the APC gene is very common in sporadic colorectal cancer, and the main inactivation mechanism of the APC gene is promoter hypermethylation. Allelic imbalance has the same frequency as mutations, and mutations in the APC gene are more common in the early stages and in tumors located in the rectum.

Software used: SIFT: http://blocks.fhcrc.org/sift/SIFT.html
PolyPhen: http://genetics.bwh.harvard.edu/pph
PMut: http://mmb.pcb.ub.es/PMUT

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Keywords Allelic imbalance · APC · Mutation · Promoter hypermethylation · Sporadic colorectal cancer

Abbreviations

APC	adenomatosis polyposis coli
AI	allelic imbalance
FAP	familial adenomatosus polyposis
MCR	mutation cluster region
SSCP	single strand conformation polymorphism
HA	heteroduplex analysis

Introduction

Colorectal cancer is the second most common malignant disease worldwide. In Hungary, it has an annual incidence of 70 cases per 100,000 inhabitants. Hereditary cases account for 5–10% of colorectal cancers, one third of which is familial adenomatosus polyposis (FAP). FAP is most frequently caused by germline mutations in the adenomatosis polyposis coli (APC) gene. The APC gene belongs to the family of gatekeeper tumor suppressor genes, and is involved in wnt signaling, cell cycle regulation, stabilization of the microtubular cytoskeleton, cell–cell interactions through β-catenin and possibly apoptosis. The APC gene encodes a large multidomain protein. The apc protein consists of an oligomerization domain and an armadillo region in the N-terminus, a number of 15 and 20 amino acid repeats in its central portion, and a C-terminus that contains a basic domain and binding sites for eb1 and the human disc large (hdlg) protein. The multiple domains of the apc protein allow it to interact with numerous protein partners. The oligomerization domain

is essential for dimer formation. The armadillo domain consists of seven repeats and shows a high degree of homology to a similar area in β-catenin. This domain is highly conserved and plays a role in stabilization and motility of the actin cytoskeleton network and is probably essential for cellular survival. The 15 and 20 amino acid repeats are binding sites for β-catenin. The 20 amino acid repeat marks β-catenin for subsequent downregulation. The apc protein binds to α- and β-catenin, which bind to E-cadherine. Cadherines are cell surface molecules that regulate the calcium-dependent intercellular interactions. If lesions occur in the β-catenin binding domain of the APC gene, cell–cell interactions get injured and the cells lose control over the cell growth and differentiation, and this could potentially promote the development of adenomas [1–3]. The basic domain of the C-terminus is probably a microtubule binding site, and truncated apc protein found in colorectal cancer seldom retain this domain. The eb1 protein is closely associated with centromeres, mitotic spindles and microtubules at all stages of the cell cycle, therefore it is involved in a checkpoint mechanism in the cell cycle. The eb1 protein directs apc to the microtubule tips [4].

The APC gene is located on 5q21 chromosomal locus. It is more than 300 kilobases, with a coding region of 8,532 bp spreading over 15 exons. There are two main mRNA sequences produced by alternative splicing. The more common sequence is longer by 300 bp and codes for a protein of 2,843 amino acids. The expression of this splice variant is regulated by the 1A promoter. In case this promoter becomes inactive, the 1B promoter takes over, expressing a protein that lacks exon 1 and some regions of the 5′ UTR [5–9]. Gene silencing through promoter hypermethylation is a well known inactivation mechanism of the APC gene beside small insertions or deletions affecting only some nucleotides or gross deletions affecting some exons, which is not typical in sporadic cases. More than 80% of the missense or nonsense mutations are located in a mutation cluster region (MCR) between codon 1,285 and 1,465 in exon 15 [1, 10–13].

The aim of this study was to determine the rate of various somatic inactivation mechanisms, including allelic loss, mutations and epigenetic inactivation such as promoter hypermethylation of the APC gene in sporadic colorectal cancer cases in Hungary.

Materials and Methods

Patients and DNA Samples

Seventy patients (34 men, 36 woman) with colorectal cancer were selected at the National Institute of Oncology, Budapest, Hungary. The patients had no family history of any type of cancer. The mean age of onset was 64 years (31–87). The tumor stages were 7 A, 34 B and 29 C by Dukes' classification. Twenty-seven and 43 tumors were located in the rectum and in the colon, respectively. Tumor and corresponding normal fresh frozen tissues were selected and isolated. DNA was extracted after proteinase K (Sigma, St. Louis, MO, USA) digestion using the standard phenol-chloroform method [14].

Promoter Methylation Assay

The assay was performed for the 1A promoter of the APC gene by bisulfite modification technique described by Frommer [15]. After bisulfite modification the methylation level of the APC promoter was measured by Taqman assay with primers and probes published by Eads et al [16] using ABI 7900 sequence detection system (Applied Biosystems, Forster City, CA, USA). Actin-β was used as a methylation-independent internal control with no CpG sites in the PCR region [16]. The methylation level was quantitated by comparing the methylation status to an unmethylated sperm control and a fully methylated artificial control. The promoter was considered hypermethylated when the tumor sample showed at least a 5% higher methylation level than the corresponding normal DNA [16].

Analysis of Allelic Imbalance

Paired tumor and normal DNA was used for testing allelic imbalance (AI), considering a threshold of 50% reduction as significant [17]. The primers for the D5S346 dinucleotide marker were fluorescently labeled, and the analysis was performed using ABIPRISM 310 genetic analyzer with GeneScan software (Applied Biosystems, Forster City, CA, USA) [18]. If microsatellite instability occurred at the locus or the locus was homozygous, the result was scored as non-informative.

PCR Reaction and Single Strand Conformation Polymorphism (SSCP)/Heteroduplex Analysis (HA)

The mutation cluster region (MCR) of the APC gene between codons 1285 and 1465 was analyzed. Primer sequences and cycling conditions were described earlier [19, 20]. After the denaturation of the PCR products gel electrophoresis was performed on 16 cm long slab gels (Hoefer SE 600 Series, Pharmacia Biotech Inc, San Francisco, CA, USA), and visualized by silver staining.

DNA Sequencing

DNA sequencing of the purified PCR products showing altered migration patterns by SSCP or HA was performed

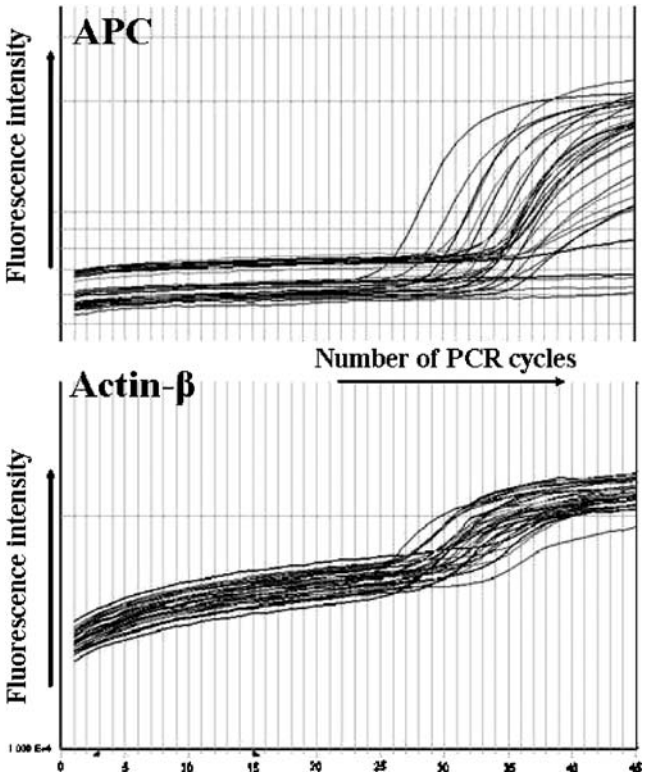

Fig. 1 Promoter hypermethylation analysis of the APC gene by Taqman assay using actin-β as an internal control. In the case of actin-β the fluorescence of all the PCR products increases as it is a methylation-independent control, but in the case of APC only the promoter-hypermethylated PCR products show increasing fluorescence intensity. The negative control and the samples proved to be negative do not show elevation in their curves. The time elapsed to the start of the rise depends on the methylation level: samples with higher hypermethylation show an earlier increase

using BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Forster City, CA, USA) on an ABIPRISM 310 genetic analyzer (Applied Biosystems, Forster City, CA, USA).

Amino Acid Conservation Analysis

The pathogenicity of the novel amino acid changes was assessed by three different software: SIFT [21], PolyPhen [22] and Pmut [23]. The change was considered neutral if all three software defined it neutral, pathogenic if all three software found it pathogenic, and probably pathogenic if only one or two software declared it pathogenic.

Statistics

Comparison of different groups was carried out using Fisher's exact test. Differences were considered significant when the p value was less than 0.05.

Results

Methylation of the APC 1A Promoter Region

The presence of APC promoter hypermethylation was observed in 21 cases (30%). Due to the primer and probe design, only dense methylation of the promoter region manifests as hypermethylation (Fig. 1).

Frequency of Allelic Imbalance

Allelic imbalance was found in 15 patients (21%) in the D5S346 dinucleotide marker and one case was considered non-informative due to homozygosity of the marker (Fig. 2).

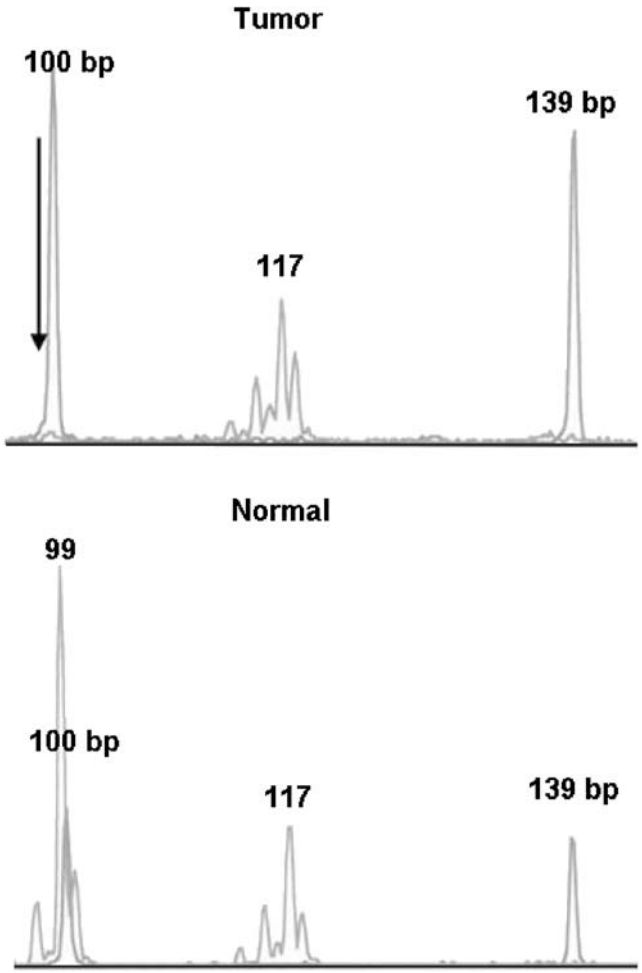

Fig. 2 Allelic imbalance analysis near the APC gene using the D5S346 marker. Tumor and corresponding normal tissue samples were analyzed. Size standards of 100 and 139 bp were used. Allelic imbalance (complete loss of an allele) is seen in patient 32. The missing allele at 99 base pairs is indicated by an arrow the in the tumor sample

Frequency of Mutations

Eighteen different somatic alterations were found by SSCP/HA followed by direct sequencing. From these alterations five proved to be missense, three nonsense, seven frameshift mutations and three polymorphisms (Table 1). Of the seven frameshift mutations six were deletions and one was insertion. In 15 patients (21%) pathogenic APC mutation was found. In four patients more than one mutation were observed (Table 1), namely, in patient 3 a missense and a nonsense, in patient 8 a frameshift and a missense, in patient 30 two nonsense, and in patient 66 two nonsense mutations were detected. From the novel non-synonymous variants the p.Met1431Arg proved to be pathogenic, the p.Ala1305Gly, the p.Ser1465Arg and the p.Leu1488Phe were considered as probably pathogenic and the p.Ala1347Val proved to be neutral by the software. From the two synonymous polymorphisms one was observed in sixteen patients and the other one in three patients. The p.Thr1493Thr polymorphism was detected in homozygous form in patient 27.

Biallelic Inactivation

Biallelic inactivation was observed in 15 (21%) patients. In four cases at least two mutations were found, in five cases (patients 2, 15, 31, 54, 57) promoter hypermethylation and mutation was detected, in five cases (patients 18, 24, 33, 41, 55) promoter hypermethylation and allelic imbalance was found and in one case (patient 48) the co-presence of allelic imbalance and mutation was observed.

Statistics

No association was found by Fisher's exact test between promoter hypermethylation and allelic imbalance (p= 0.7583) or promoter hypermethylation and mutation (p= 1). Although allelic imbalance showed negative correlation with the presence of a mutation (RR<1), the correlation was not significant (p=0.1631).

Discussion

The maintenance of cell–cell interactions is crucial to avoid unregulated cell growth. The apc protein plays an important role in cell adhesion via β-catenin. Functional disruption of this gene increases the risk for loss of cell–cell interaction, which in turn results in tumor progression. Since the APC gene is a tumor suppressor gene, both alleles should be inactivated for cancer development. These two independent events could be allelic imbalance, promoter hypermethylation or mutations.

In this study 70 sporadic colorectal cases were analyzed to determine the frequency of the inactivation mechanisms in the APC gene. Epigenetic inactivation of different tumor suppressor genes has a great importance in the development and progression of various cancers. We have shown that the frequency of APC promoter hypermethylation was 30% in sporadic colorectal cancer. This finding indicates that promoter hypermethylation is the main inactivation mechanism in the APC gene in our population. Similar results were

Table 1 Genetic alterations in the MCR of the APC gene. Novel variations are in bold

Nucleotide change	Protein change	Patient number
Mutation		
c.3792delA	**Frameshift**	7
c.3897del11	**Frameshift**	48
c.3914C>G	**p.Ala1305Gly**	2
c.3915deltgcaa	**Frameshift**	54
c.3920T>A	p.Ile1307Lys	42
c.3944C>G	p.Ser1315Stop	66
c.3982C>G	p.Gln1328Stop	30,66
c.4037C>G	p.Ser1346Stop	3,16,30,85
c.4282insccaaa	**Frameshift**	8
c.4292T>G	**p.Met1431Arg**	8
c.4395T>G	**p.Ser1465Arg**	3
c.4464A>T	**p.Leu1488Phe**	15
c.4464del11	**Frameshift**	57
c.4476delC	**Frameshift**	31
c.4488del11	**Frameshift**	49
Polymorphism		
c.4040C>T	**p.Ala1347Val**	66
c.4350A>G	**p.Arg1450Arg**	17, 19, 25
c.4479G>A	p.Thr1493Thr	5, 6, 8, 10, 13, 18, 20, 22, 27, 30, 38, 42, 49, 66, 68, 70

published previously by Arnold [24] and by Thorstensen [25]. It should be noted that our hypermethylation analysis may underestimate the frequency of inactivation of this gene, as it is positive only when all the CpG islands of the APC promoter region covered by the primers and the probe are hypermethylated. Other studies suggest that only dense methylation of the promoter region is associated with decreased mRNA expression [26]. Therefore the detection of dense methylation is the most appropriate approach for the estimation of biological inactivation of the APC gene.

Allelic imbalance was observed with the same frequency as mutations (21%). In other studies [27] allelic imbalance was observed with a similar rate (20%). From the 18 different somatic alterations 15 true somatic pathogenic mutations, one non-synonymous and two synonymous polymorphisms were observed (Table 1). The frameshift mutations, four missense mutations (p.Ala1305Gly, p.Met1431Arg, p.Ser1465Arg, p.Leu1488Phe) and two polymorphism (p.Ala1347Val, p.Arg1450Arg) have not been published before. The effect of a novel polymorphism on tumor formation is hard to determine, but its contribution to the inactivation mechanisms, especially in homozygous form, can not be ruled out. The three nonsense mutations, one missense (p.Ile1307Lys) mutation and the other polymorphism (p.Thr1493Thr) were published before. Two of them (p.Ser1315Stop, p.Ile1307Lys) are characteristic of the Ashkenazi population [28–35].

Forty patients (57%) had somatic inactivation of at least one of the alleles, and 15 patients (21%) had biallelic somatic inactivation. In four cases double mutations were observed, and three of them also carried a polymorphism. Five of the cases had promoter hypermethylation together with allelic imbalance, five other patients carried mutations coupled with promoter hypermethylation. Only one person showed both a mutation and allelic loss. We found neither positive [36] nor inverse [37] association between promoter hypermethylation and allelic imbalance or mutation. Allelic imbalance is negatively, but not significantly correlated with mutations in the APC gene. The same inverse correlation, though in a significant manner was published by Thorstensen, although other studies suggest positive association between allelic imbalance and mutation [34, 38]. Genetic and epigenetic inactivation seems to occur in the same tumor, but allelic imbalance and mutation may represent distinct inactivational pathways. The mutations were found to occur more frequently in cancers located in the rectum than in those in the colon, which is similar to another author's findings [39]. Mutations were also more common in cases with Dukes' A or B stages than in Dukes' C stage as it was published by DeFilippo [39].

In conclusion, these results suggest that the inactivation of the APC gene in sporadic colorectal cancer can be attributed to the combination of various genetic mechanisms. Our data suggest that the main inactivation mechanism in the APC gene is promoter hypermethylation. Allelic imbalance and mutations cause inactivation with a similar frequency. Mutations in the APC gene occur more frequently in the early stages and in tumors located in the rectum.

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