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Mutations at the *APC* exon 15 in the colorectal neoplastic tissues of serial array

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Abstract Although the APC protein is known to participate in cellular proliferation and apoptosis, *APC* mutations have been thought to play a major role in the early stage of colorectal tumorigenesis. The somatic *APC* mutation of exon 15 was assessed to determine its impact on various stages of colorectal tumorigenesis. The colorectal neoplastic tissues of serial array studied included sporadic adenomas (group 1, *n*=36), adenomas (group 2, *n*=33), and carcinomas (group 3, *n*=32) in the synchronous adenoma and carcinoma as well as sporadic carcinomas (group 4, *n*=36). Aberrant DNA was detected by protein truncation test and confirmed by direct sequencing. The mutation prevalence was 36.1% in group 1, 45.5% in group 2, 59.4% in group 3, and 41.7% in group 4 with no differences among the groups. Among the 18 patients with synchronous adenoma and carcinoma, 9 had mutation in their adenomas and 12 in their carcinomas. The mutation loci and patterns did not differ in adenomas and carcinomas. Mutations in the mutation cluster region (MCR) were much more frequent than in the preceding region of MCR, i.e., 85.7% vs. 14.3%. The mutation prevalence of villous adenomas appeared greater than that of tubular adenoma (3/21 vs. 3/4). Predominant pathogenic mutations at MCR suggest that the *APC* mutation is implicated in all stages of colorectal tumorigenesis.

Keywords *APC* · Exon 15 · Colorectal adenoma · Colorectal carcinoma

Introduction

Colonic polyp formation is known to occur when the level of APC protein activity falls to critical or threshold value. The major defect in preneoplastic intestine harboring *APC* mutation is reported to accelerate crypt fission, which is also the mode by which microadenoma enlarges [1]. It is accepted that *APC* mutation plays a major role in the early stage of colorectal tumorigenesis [2, 3, 4]. This hypothesis is based mainly on the observation of a similar incidence of *APC* mutation between adenomas and carcinomas.

The APC protein participates in cellular proliferation and apoptosis probably directly affecting the cell cycle [5]. It down-regulates β-catenin and results in transcriptional inactivation in association with glycogen synthase kinase 3β (GSK3β) and axin [6]. In addition, it directly links to the cytoskeleton and plays a role in the migration of epithelial cells stabilizing microtubules. These properties appear to provide colonic epithelial cells with more than a single gatekeeper function. De Benedetti et al. [3] studied 59 sporadic colonic adenomas and suggested that the *APC* mutation confers a growth advantage on small adenoma through expansion of villous components. New *APC* mutations may also be acquired after the development of a superficial depressed adenoma as observed in various types of adenoma as well as submucosal and advanced carcinomas [7]. These studies and the various functions of APC protein suggest the implication of *APC* mutation in both the early and late stages of colorectal tumorigenesis

This study was performed to identify the somatic *APC* mutation of exon 15, known to include important domains, in colorectal neoplastic tissues of serial array. The prevalence and patterns of mutations were assessed to determine the impact of *APC* mutation during various stages of colorectal tumorigenesis.

Materials and methods

Tumor samples

Fresh colorectal tumor tissue was obtained from surgical or biopsy specimens: group 1 (n=36), adenomas from sporadic adenoma; group 2 (n=33), adenomas from synchronous adenoma and carcinoma; group 3 (n=32), carcinomas from synchronous adenoma and carcinoma; and group 4 (n=36), carcinomas from sporadic carcinoma. All tumors were acquired from patients with sporadic colorectal tumors excluding those with familial adenomatous polyposis and hereditary nonpolyposis colon cancer. Both adenoma and carcinoma tissue was simultaneously available in 18 patients from groups 2 and 3. Histologically confirmed neoplastic tissue was included with the DNA extracted using standard methodology

Protein truncation test

We used the protein truncation test (PTT) to identify the translation-terminating mutation in exon 15 [8]. Four primer pairs were used to amplify whole reading frames of exon 15, including overlapping base sequences of 1959–3789, 3084–5100, 4782–7009, and 6300–8668, respectively. The polymerase chain reaction (PCR) was started with a denaturation cycle of 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C. PCR products were introduced into the TnT T7 rabbit reticulocyte system (Promega, Southampton, UK) with ^{35}S incorporation (Amersham, Buckinghamshire, UK). Products were resolved onto a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized on autoradiography.

Single-strand conformation polymorphism analysis

Aberrant bands identified on PTT were also verified by single-strand conformation polymorphism analysis for efficient sequencing. The primers for PCR amplification and methods were derived from Miyoshi et al. [9]. PCR products were heated to 90°C for 5 min and quenched on ice. Electrophoresis was performed in 10% nondenaturing polyacrylamide gels with an acrylamide: bisacodylamide ratio of 30:0.8 containing 10% glycerol at 20 mA for 18 h. DNA bands of distinct mobility were detected by silver staining

Direct sequencing

Aberrant electrophoretic mobility on single-strand conformation polymorphism analysis was determined by direct sequencing. PCR products were sequenced using the dideoxy chain-termination reaction with a Sequenase version 2.0 kit (Amersham). All sequencing reactions were performed in duplicate and any indeterminate results were also verified using the ABI sequencing kit on an ABI 377 Prism sequencer (Perkin-Elmer, Norwalk, Conn., USA).

Statistical analysis

The results of mutation loci and patterns were analyzed regarding respective groups using Fisher's exact test. A mean number of mutation and adenoma were tested by analysis of variance using Scheffe's test. The presence of mutation was also verified regarding age, sex, and family history by multiple regression analysis. The significance level was set at 5% for each analysis, and all calculations were performed on an IBM PC using Statistica (version 5.1, StatSoft, Tulsa, Okla., USA).

Results

Approximately 45% of samples showed mutations at exon 15 (Table 1). The mutation prevalence did not differ significantly between the groups. All except for three samples (numbers 39, 18, and 243) included both wild-type and truncated protein segments (Fig. 1). The mean age was less in patients with neoplastic tissue including mutation than in those without it (56±11 vs. 60±9; P=0.016). The age difference was more prominent in group 2 than in the other groups. In group 3 a family history of colorectal or other cancers was more frequently associated in patients with mutation than those without. Of 11 patients with a family history of cancer 10 (90.9%) had mutation, as opposed to 9 of 21 (42.9%) without a family history cancer (P=0.009). However, the presence of mutation was not significant in the multiple regression analysis with confounding variables, namely age, sex, and family history. The number of mutations per tissue did not differ between the groups: group 1, 1.2±0.4; group 2, 1.5±1.1; group 3, 1.0±0.0; and group 4, 1.4±0.6

All translation-terminating mutations were found in the 5′ half of exon 15. All mutations occurred between codons 893 and 1512 which occupied approximately 32% of exon 15 (Fig. 2). If the mutation locus was divided at codon 1250 (codons 893–1249 and 1250–1512) near the beginning of the mutation cluster region (MCR),

Table 1 Somatic mutations at exon 15 of *APC* in respective groups (differences nonsignificant)

	Total no. of samples	Samples with mutation	
		n	%
Adenomas from sporadic adenoma	36	13	36.1
Adenomas from synchronous adenoma and carcinoma	33	15	45.5
Carcinomas from synchronous adenoma and carcinoma	32	19	59.4
Carcinomas from sporadic carcinoma	36	15	41.7

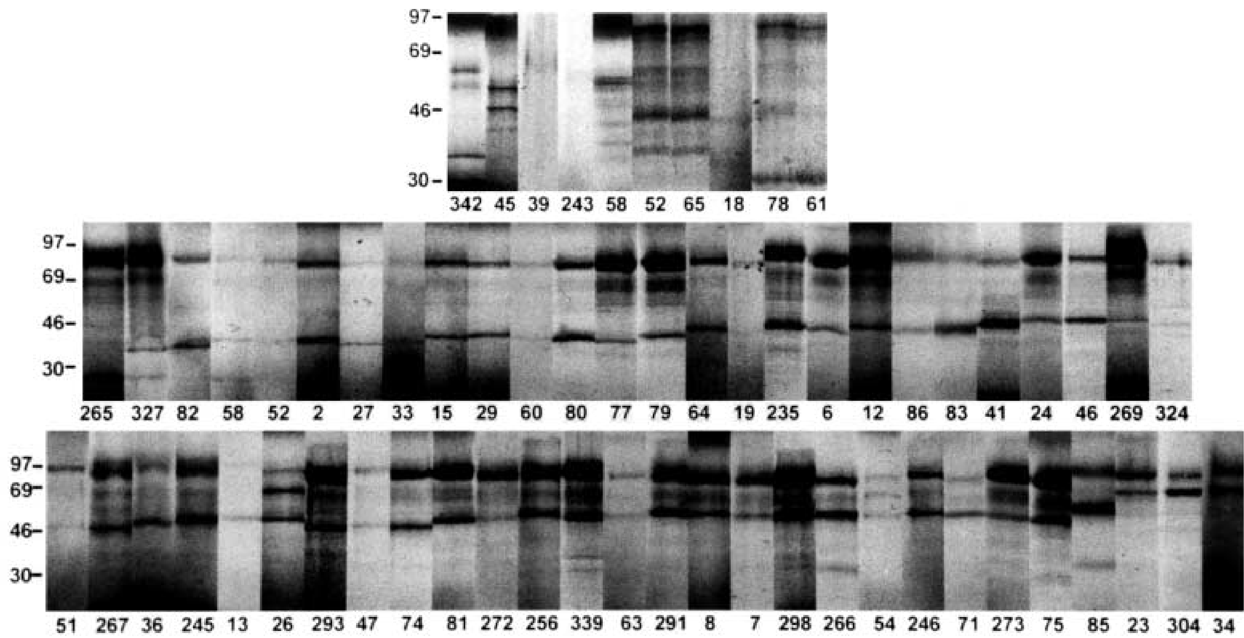

Fig. 1 Protein truncation test results of all samples using primers identifying two different overlapping segments. Upper autoradiogram including codons 653–1269; middle and lower autoradiogram including codons 1028–1700

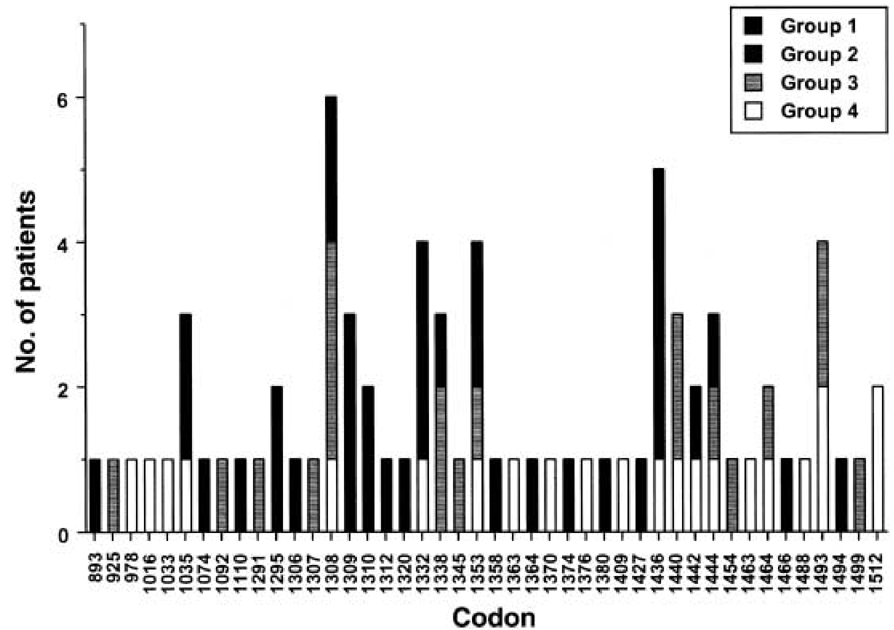

Fig. 2 Somatic mutations at exon 15 of *APC* in the colorectal neoplastic tissues of serial array

mutations in the latter loci were much more frequent than in the former loci (85.7% vs. 14.3%; $P<0.001$). This difference was significant in all groups. All mutation patterns identified on PTT were translation-terminating mutations, namely nonsense, insertion, and deletion, consisting of 39%, 35.1%, and 26%, respectively (Table 2, Fig. 3). Although nonsense mutation was frequently found in groups 2 (47.8%) and 3 (52.6%), mutation patterns did not differ significantly between the groups. Interestingly, these patterns were different between patients with and without a family history of colorectal or other cancers. Insertion was more prevalent in patients without a family history than those with a family history (45.5% vs. 9.1%), while nonsense (54.5% vs. 32.7%) and deletion (36.4% vs. 21.8%) occurred more frequently in patients with a family history ($P=0.01$)

The mutation loci and patterns did not differ between adenomas and carcinomas. Mutations were identified in

Table 2 Summary of somatic mutations at exon 15 of *APC*

Affected codon	Mutation	No. of samples with mutation
Nonsense		
978	2932C→T	1
1291	3871C→T	1
1295	3883G→T	2
1306	3916G→T	1
1308	3922A→T	3
1309	3925G→T	2
1310	3928A→T	1
1312	3934G→T	1
1338	4012C→T	3
1345	4035G→T	1
1353	4057G→T	4
1374	4120G→T	1
1444	4330C→T	3
1454	4360A→T	1
1463	4387A→T	1
1464	4390G→T	2
1466	4396G→T	1
1488	4463T→A	1
Deletion		
925	2773, 2774delAG	1
1016	3048delT	1
1074	3222delT	1
1110	3328delT	1
1307	3919delA	1
1308	3922delA	3
1309–1310	3925del5	1
1320	3960delG	1
1332	3994delA	4
1358/1364	4072, 4074, 4091delG	1
1409	4227delA	1
1436	4308delT	1
1440	4320delA	3
Insertion		
893	2679insC	1
1033	3098insG	1
1092	3275insG	1
1363/1370	4089, 4109insT	1
1376	4126insC	1
1380	4140insG	1
1427	4279insA	1
1436	4307insT	4
1442	4325insG	2
1493	4479insA	4
1494	4480insA	1
1499	4497insT	1
1512	4534insT	2

23 of 41 neoplastic tissues (56.1%) from 18 patients from groups 2 and 3 who had synchronous adenoma and carcinoma. Among these, 9 had mutation in their adenomas, while 12 had it in their carcinomas. Seven patients had mutation in both their adenomas and carcinomas. The mutation loci and patterns between the adenoma and carcinoma were consistent in two patients who showed nonsense mutations of K1308X and Q1444X. Four patients had no mutation in either their adenoma or carcinoma

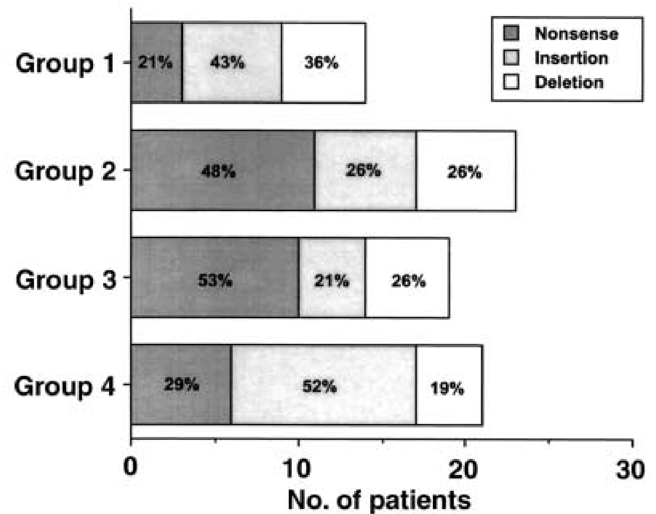

Fig. 3 The mutation patterns at exon 15 of *APC* in respective groups

Although our sample was not sufficiently large to allow us to reach a definite conclusion, the mutation prevalence of villous adenomas appeared greater than that of tubular adenoma in group 2 (3/24 vs. 3/4, *P*=0.005). However, location, size, number, gross morphology (sessile or pedunculated), and degree of dysplasia were not associated with prevalence of mutation in groups 1 and 2. The number of mutations in neoplastic tissue was not correlated with the degree of dysplasia or the histological types. Similarly, the TNM stage, location, and tumor differentiation was not correlated with prevalence of mutation in either groups 3 or group 4

Discussion

The incidence of somatic *APC* mutation was reported as 47%–83% in colorectal neoplasm [2, 3, 4]. Miyaki et al. [10] reported that more than 95% of the somatic *APC* mutation occurred in exon 15 among 241 colorectal tumors in patients with or without familial adenomatous polyposis. As our study did not include the missense mutation and mutations in exon 1–14, our incidence of 45% appeared to be less than in previous investigations. Among 18 subjects in groups 2 and 3 with both adenoma and carcinoma tissue available, 9 adenomas and 12 carcinomas showed mutation. Although the difference seems to be insignificant between adenoma and carcinoma, these findings suggest that an additional or new *APC* mutation in carcinoma precedes the late stage of carcinogenesis. An inconsistency of mutations between adenomas and carcinomas may also suggest this possibility. One recent study reported a respective incidence of *APC* mutation of 7% and 35% in 15 superficially depressed adenoma and 19 flat early carcinomas without mentioning the significance [7]. Some researchers have re-

ported concordance of *APC* mutation at a different site or in the same adenoma with a different degree of dysplasia suggesting accumulation rather than order of *APC* mutation during progression to late stage adenoma [3].

The APC protein identified on PTT showed both wild-type and truncated proteins in most samples of this study. Tumor samples are prone to contain a significant portion of wild-type DNA from normal stromal or epithelial tissue. Otherwise, as all tissues were cosegregated with neoplastic lesions, a dominant-negative effect seemed to be activated for tumorigenesis. Frequent single-basepair deletion and insertion in our study were similar to preceding results indicating the prevalence of single base-pair deletion at 21.5–33% and that of small insertion at 9–42% [4, 10, 11]. Of 42 codons 25 (59.5%) were the same mutation sites as in previous reports (HGMD, http://www.uwcm.ac.uk). However, the same mutation was found in only 6 of 42 mutations. The mutation loci and patterns were not consistent between adenoma and carcinoma tissues from the same subjects. These inconsistencies suggest the possibility of new somatic mutations along the MCR which appear to have emerged from the impact of an environmental mutagen. G→T substitution was the most frequent (9/18), and this is known as the most common DNA polymerase error, showing a lack of repair at this mismatch [9, 12]. This misparing may provide frequent nonsense mutations in patients with a family history of any cancer. However, the greater prevalence of deletion and insertion in these patients still must be clarified. Two nonsense mutations, K1308X and Q1444X, were novel mutations simultaneously found in both adenoma and carcinoma tissues from the same subjects. Further identification is needed to confirm their significance in colorectal carcinogenesis.

Mutations in villous adenomas were more frequent than in tubular adenomas as is consistent with other reports [3, 13]. One study reported that 10 of 13 tubulovillous or villous adenomas showed *APC* mutation in contrast with 15 of 45 in tubular adenomas [3]. Size, location, and degree of dysplasia were not correlated with *APC* mutation in our study. These findings still conflict between various studies [2, 3, 13, 14]. As the size and the extent of the villous component are known to be major independent risk factors associated with high-grade dysplasia [15], paths other than the *APC* mutation may exist for those risk factors without positive correlations. The number of *APC* mutations was not correlated with the degree of dysplasia or the histological types in our study, while a close association has been identified in some studies [4, 10]. Thus the dose effect being dependent on the number of mutations does not appear to be consistent in the progression to high-grade dysplasia or villous adenoma. Some researchers have found that loss of heterozygosity of 5q21 is more frequent in adenoma with high-grade rather than in low-grade dysplasia [10, 16].

Although 80% of primary colorectal tumors are thought to contain *APC* mutations along the entire exons [4], mutations upstream of exon 15 are not thought to be advantageous to tumor development [17]. It has been proposed that mutation at the extreme 5′ end of the gene presumably lead to unstable heterodimers of mutant with wild-type APC protein, thus providing a sufficient level of active wild-type homodimers [18, 19]. Mutations in MCR were usually more frequent than those in the preceding codons, similar to earlier reports [9, 10]. Although MCR represents less than 10% of the entire reading frame of *APC*, it encompasses three sites (codons 1262–1281, 1376–1395, 1492–1511) of several β-catenin down-regulating repeats [20, 21]. Truncation at this site also produces inactivation of the carboxyl terminus region, which appears to be important in adenoma development [22]. The APC protein is known to involve an integral part of the signaling pathway by complex formation with GSK3β, β-catenin, axin, and conductin [23]. It also regulates intercellular adhesion and migration which determines tumor progression in association with β-catenin and E-cadherin [24].

Prevalent mutations on the MCR of *APC* in both colorectal carcinomas and adenomas can critically impair the APC protein function interacting with β-catenin and other relevant proteins. These impairments may implicate the entire cell cycle irrespective of a variety of tumors and an additional or new *APC* mutation in carcinoma may precede the late stage of carcinogenesis.

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