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Lack of mutations of the adenomatous polyposis coli gene in oesophageal and gastric carcinomas

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Abstract The adenomatous polyposis coli (*APC*) gene is the target of the loss of chromosome 5q heterozygosity observed frequently in gastrointestinal tract carcinomas and is inactivated in these carcinomas. We screened 94 gastrointestinal tract carcinomas for *APC* mutations, by polymerase chain reaction single-strand conformation polymorphism (SSCP) analysis. Mutations were detected in 8 of 21 (38%) colorectal carcinomas in the mutation cluster region of the *APC* gene whereas no mutation was detected in any of 49 oesophageal and 24 gastric carcinomas, even though SSCP analysis was extended to include the 5′ half of the *APC* gene exon 15. Direct DNA sequencing revealed that six of eight (75%) mutations in colorectal carcinomas resulted in truncated gene products. These findings confirm the significance of *APC* gene mutations in colorectal, but not oesophageal or gastric carcinomas. Some other tumour suppressor genes near the *APC* gene may be the target of the frequent allelic loss of chromosome 5q in oesophageal and gastric carcinomas.

Key words Adenomatous polyposis coli gene
Gastrointestinal tract carcinoma · Mutation
Polymerase chain reaction · Tumour suppressor gene

Introduction

Frequent allelic deletions at specific chromosomal loci imply that one or more tumour suppressor genes exist on each chromosome in many human malignancies [1, 13, 19, 20, 24]. Loss of heterozygosity (LOH) involving chromosome 5q has been examined in oesophageal [1, 6, 11], gastric [12, 19, 23], colorectal [14] and pancreatic carcinomas [12].

The adenomatous polyposis coli (*APC*) gene has been isolated and mapped to 5q21 [9]. Germline mutations of the *APC* gene have been found in patients with familial adenomatous polyposis (FAP) [2, 7, 14, 16, 17, 25] and somatic mutations of this gene were detected frequently in colorectal carcinoma [2, 13, 16, 18] and in some gastric carcinomas [5, 15]. These observations suggest that the *APC* gene is most likely to be the target of frequent 5q-LOH and may play a significant role in the pathogenesis of the great majority of gastrointestinal tract carcinomas [4, 5, 15]. However, mutation of this gene has not yet been evaluated in oesophageal carcinoma. Since there is also a great disparity in prevalence between mutations [4, 5, 15] and LOH [12, 19, 23] in gastric carcinoma, it is not clear whether the *APC* gene is the true target of this 5q-LOH. In addition, it has been demonstrated recently that most mutant *APC* proteins can bind to wild type *APC* protein, suggesting that the mutant proteins inactivate the wild one in a dominant negative manner [21]. We therefore have examined somatic mutations of the *APC* gene in 94 carcinomas arising from the oesophagus, stomach and colorectum. As the entire coding region of the *APC* gene is too long (8538 bp) to permit mutations to be analysed exhaustively in a large series of patients, we chose the polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) analysis for the present screening for *APC* mutations [25].

Materials and methods

Tumour specimens obtained surgically from 94 patients consisted of 49 oesophageal squamous cell carcinomas (SCC), 24 gastric and 21 colorectal carcinomas. Oesophageal and gastric carcinomas were previously analysed for LOH at the APC locus, which revealed that 86% (12/14 informative cases) of the gastric and 55% (16/29) of oesophageal carcinomas had lost heterozygosity at this locus [11, 23]. The colorectal carcinomas studied were sporadic cases, and no FAP patients were included. Table 1 summarizes their histopathology. The samples were frozen immediately and stored at −80° C until use for genic analysis. DNA was isolated by standard proteinase K digestion and phenol/chloroform extraction, from oesophageal and colorectal carcinoma specimens. Gastric carcinoma DNA was isolated after tumour cell enrichment by cell

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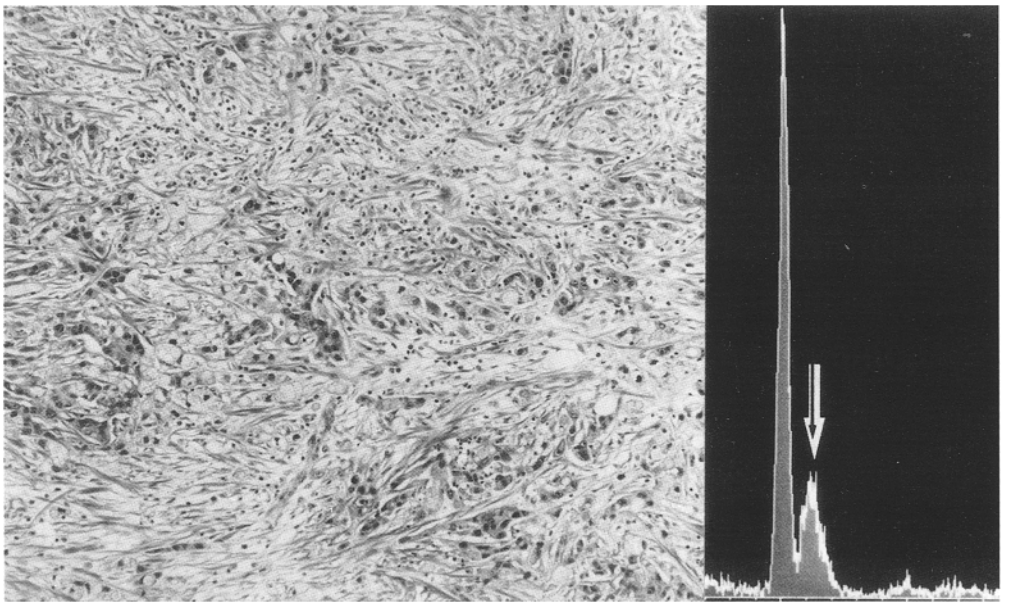

Fig. 1 Histological appearance of poorly differentiated adenocarcinoma of the stomach (*left*, haematoxylin and eosin, ×100) and DNA histogram of an aneuploid tumour (*right*). A large number of lymphocytes and fibroblasts had infiltrated the tissue. The aneuploid cell population, which is indicated by the *arrow* in the histogram, was isolated

Table 1 Histopathological types of 94 carcinomas

Oesophageal squamous cell carcinoma:	49 cases
well differentiated	19
moderately differentiated	22
poorly differentiated	8
Gastric adenocarcinoma:	24 cases
differentiated	15
undifferentiated	9
(involving 3 cases of signet-ring cell carcinoma)	
Colorectal adenocarcinoma:	21 cases
well differentiated	11
moderately differentiated	9
poorly differentiated	1

sorting, an extremely sensitive method for detecting genetic abnormalities that avoids heavy contamination by normal cell DNA [10, 22, 23] (Fig. 1). The procedure was as follows: nuclei were isolated with 0.2% Triton X-100 (Sigma, St. Louis, Mo.), treated with 0.1% RNase (Sigma), stained with 50 μg/ml propidium iodide (Sigma) and filtered through a 40 μm diameter nylon mesh. DNA flow cytometry and cell sorting were performed using a FACStar Plus (Becton Dickinson, Mountain View, Calif.). Aneuploid tumour cells (Fig. 1) and diploid tumour cells in S and G_2 M phases were sorted. DNA was isolated from 1–5× 10^4 nuclei.

For PCR-SSCP analysis the 5′ half of exon 15 (codons 653–1673) of the APC gene was divided into 8 segments which included the mutation cluster region (MCR) of colorectal carcinoma cells [13], and amplified by PCR using primers designed by Miyoshi et al. [14] which are listed in Table 2. All 8 segments were examined in oesophageal SCC and gastric carcinoma specimens, and two segments (segment numbers 6 and 7), corresponding to the MCR, were examined in colorectal carcinoma specimens. One hundred nanograms of genomic DNA was amplified in a total volume of 10 μl in the buffer recommended by Perkin-Elmer Cetus (Norwalk, Conn.). PCR was performed with 40 cycles for 0.5 min at 95° C, 2 min at 51° C, and 2 min at 70° C. The PCR products were labelled with [α_{32}P]dCTP (3000 Ci/mmol, 10 Ci/ml) during PCR. A 5 μl volume of the PCR product was diluted with 45 μl of a gel-loading buffer (98% deionized formamide, 10 mM ethylenediaminetetracetic acid pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue), denatured at 94° C for 2 min, and kept on ice until analysis on a 6% neutral polyacrylamide gel. Electrophoresis was performed at 40 W for 4 h at 4° C. The gel was then transferred to Seq gel filter paper (Bio-Rad, Calif.), dried on vacuum slab gel dryers, and exposed to X-ray film (XAR, Kodak, Tokyo, Japan) at −80° C for 2 h.

The shifted bands detected by SSCP analysis were separated from the other PCR products, eluted from the polyacrylamide gel with 25 μl of distilled water at 80° C. After brief centrifugation, a 5 μl aliquot was amplified by PCR using the same primers and protocols as described previously for PCR-SSCP analysis. The PCR products were purified using a DNA trapping cell (DNA C,

Table 2 Oligonucleotide primers[a] (5′–3′)

Segment number	Codons	Upstream	Downstream
1	653– 751	CAATCATATTATGCCTTTTGTC	GATGGCAAGCTTGAGCCAG
2	735– 884	CGAAGTACAAGGATGCCAAT	CAGTGGTGGAGATCTGCAA
3	862–1022	AACTACCATCCAGCAACAGA	TCTAGTTCTCCATCATTATCAT
4	998–1141	TCAATACCCAGCCCGACCT	GGCTTATCATCTTCATAGTCA
5	1125–1284	GTAAGCCAGTCTTTGTGTC	CAGCTGATGACAAAGATGAT
6	1260–1410	AGACTTATTGTGTAGAAGATAC	ATGGTTCACTCTGAACGGA
7	1389–1547	TCTGTCAGTTCACTTGATAG	CATTTGATTCTTTAGGCTGC
8	1516–1673	ACAGAAAGATGTGGAATTAAG	TTCTCCAGCAGCTAACTCAT

[a] These primers were designed by Miyoshi et al. (1992b)

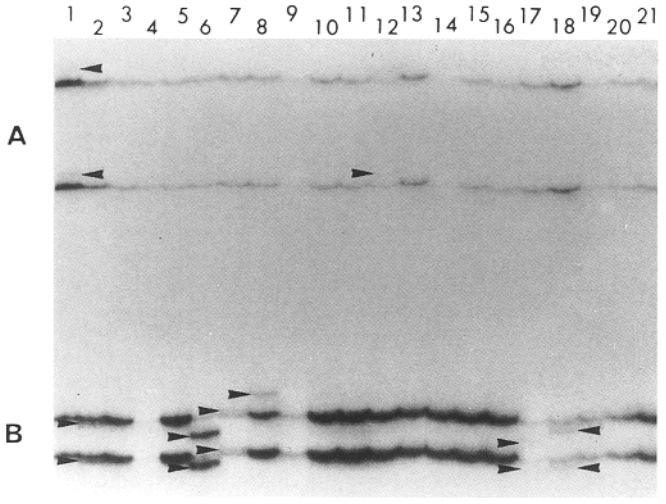

Fig. 2 Polymerase chain reaction single-strand conformation polymorphism analysis of segments 6 (**A**) and 7 (**B**) of the adenomatous polyposis coli gene in 21 colorectal carcinomas. Mobility shifts (*arrowheads*) were detected in patients 1, 2, 6, 7, 8, 12, 17 and 18

Table 3 Mutations of the adenomatous polyposis coli gene in colorectal carcinomas

Patient number	Codon	Nucleotide changes	Amino acid changes
1	1309	GAA to TAA	Glu to stop
2	1398	AGTCG to TCG	AG deletion
6	1380	ACC to AACC	A insertion
7	1376	TAT to TTAT	T insertion
8	1378	CAG to TAG	Glu to stop
12	1425	GAT to GCT	Asp to Ala
17	1367	CAG to TAG	Glu to stop
18	1364	AGT to AGG	Ser to Arg

Daiichi Pure Chemicals, Tokyo, Japan) and sequenced using a terminator cycle sequencing kit (*Taq* DyeDeoxy, Applied Biosystems, Calif., USA) and a DNA sequencer (type 373A, Applied Biosystems), with the same primers as those used for PCR.

Results

Mutations of the *APC* gene were detected in 8 of 21 (38%) colorectal carcinoma specimens in the MCR of the *APC* gene (Fig. 2), whereas no mutation was detected in 49 oesophageal SCC and 24 gastric carcinomas in any of the 8 *APC* gene segments examined. Direct DNA sequencing confirmed that six of the eight (75%) mutations detected in the colorectal carcinomas resulted in truncated gene products (Fig. 3). Table 3 shows the sequencing results.

Discussion

It has been shown recently, using RNase protection analysis followed by DNA sequencing, that over 60% of the

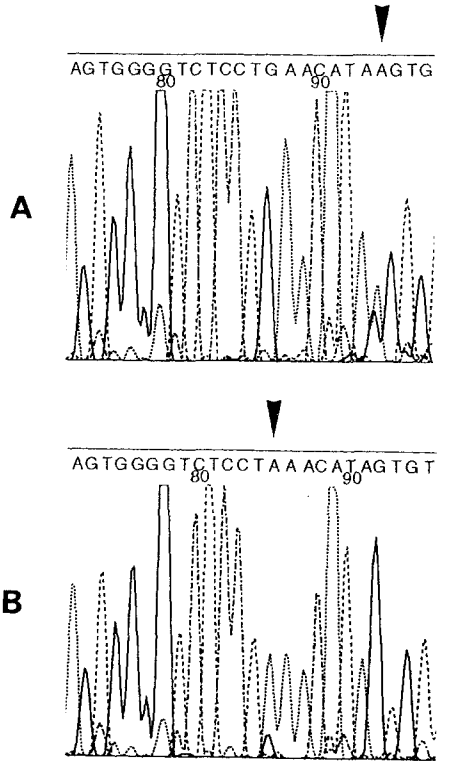

Fig. 3 Automated fluorescent DNA sequencer histograms of segment 6 using the anti-sense primer in patients 7 (**A**) and 8 (**B**). Mutations are indicated by *arrowheads*. A T insertion at codon 1376 in patient 7 and a point mutation (C to T) at codon 1378 in patient 8 are shown

somatic *APC* mutations of sporadic colorectal carcinoma are clustered within the small region of exon 15, designated as the MCR, which comprises less than 10% of the coding region [13]. Most mutations resulted in truncation of the *APC* gene product. Therefore, the mutations may be associated with development of the great majority of colorectal carcinomas [13]. The present study may underestimate the true prevalence of *APC* mutations because SSCP is not sufficiently sensitive to detect every sequence change [25]. However, we found the incidence (38%) of *APC* mutations limited to the MCR in colorectal carcinoma as detected by PCR-SSCP analysis to the almost equal to that determined by Miyoshi et al. (19/47, 40%) [13] using RNase protection analysis and by Powell et al. (10/21, 48%) [18] using PCR DNA direct sequencing. No mutation was detected among 49 oesophageal SCC and 24 gastric carcinomas, although SSCP analysis extended to about a half of the *APC* gene exon 15 or 40% of the entire coding region. Our present findings suggest that, in contrast to colorectal carcinoma, *APC* gene mutations are absent or at least rare in most oesophageal SCC and gastric carcinomas. Thus, *APC* gene mutations may not contribute in a uniform way to the pathogenesis of carcinomas arising from the gastrointestinal tract.

Nakatsuru et al. [15] reported that the *APC* gene is mutated frequently in two particular histopathological types: very well differentiated adenocarcinoma (7/17,

41%) and signet-ring cell (undifferentiated type) carcinoma (3/10, 30%). No mutation could be detected in poorly differentiated adenocarcinoma specimens (0/11) and mutations were rare in well or moderately differentiated adenocarcinoma (2/19, 11%). The incidence of mutations may be very low among gastric carcinomas of the major histopathological types. Our series included no very well differentiated type carcinoma and only three signet-ring cell carcinomas.

Horii et al. [4] detected frequent *APC* mutations in pancreatic carcinoma but not in renal cell carcinoma, hepatocellular carcinoma or carcinoma of the lung which exhibit frequent 5q-LOH. In the present study, *APC* mutations were not detected in any of 49 oesophageal SCC or 24 gastric carcinomas, although many specimens exhibited LOH at this locus (12/14, 86% of gastric carcinoma) [23] (16/29, 55% of oesophageal SCC, [11]. It is possible that other domains of the *APC* gene are associated with these carcinomas as we did not examine the entire coding region. However, some other tumour suppressor gene near but distinct from the *APC* gene may play a major role in oesophageal and gastric carcinogenesis similar to that in carcinomas of the lung, kidney and liver as proposed previously by Horii et al. [4].

We reported previously that LOH at the APC gene locus is always accompanied by LOH at the mutated in colorectal cancer (MCC) gene locus in oesophageal and gastric carcinomas [11, 23]; the genes are both located on 5q21. Therefore, MCC is a candidate tumour suppressor gene in oesophageal and gastric carcinomas. Other tumour suppressor genes were previously suggested to be present on 5q [3, 8] and further studies are required to identify the major target of the frequent 5q loss observed in oesophageal and gastric carcinomas.

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