A Very Large Villous Adenoma with an Adjacent Cancer of the Rectum: An Informative Case for Testing the Proposed Molecular Basis of Colorectal Tumorigenesis

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It is currently accepted that colorectal tumorigenesis results from accumulation of multiple mutations in certain genes. This concept prompted us to search for possible mutations in the APC, k-ras, and p53 genes in an advanced cancer coexisting with a large villous adenoma of the rectum in a 54-year-old patient with no family history of colorectal cancer. Genomic DNA extracted from multiple subregions of the tumor and surrounding normal mucosa was studied by polymerase chain reaction (PCR) followed by single-strand conformation polymorphism (SSCP) analysis and direct sequencing. Both the adenoma and carcinoma had abnormal PCR-SSCP for APC (exon 11) and k-ras, irrespective of the location within the tumors. However, p53 abnormality (exon 7) was detected only in samples taken from the carcinoma. Subsequent sequencing revealed a TTG to TAG mutation at codon 479 of APC, a GGT to GAT mutation at codon 12 of k-ras in both the adenoma and carcinoma, and a CGG to TGG mutation at codon 248 of p53 (exon 7) in the carcinoma. These findings were in accord with the current concept of colorectal tumor progression whereby genetic alteration of APC and k-ras occurs relatively early while that of p53 is rather late and is possibly a decisive event in relation to malignancy.

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Key words: Villous tumor—Adenoma-carcinoma sequence—APC—k-ras—p53

Introduction

It is currently accepted that the accumulation of multiple gene mutations underlies the process of multistep tumorigenesis in the colon and rectum.¹,² In this genetic model, it is proposed that mutations of the APC gene and possibly k-ras occur early in adenoma formation, whereas those of the p53 and DCC genes are rather late events.¹,²

Villous adenoma is an adenomatous type of colonic polyp, which is distinct from a tubular adenoma in its occurrence, morphology, and behavior. Villous adenomas constitute only about 4–10% of all adenomatous polyps in the colon and rectum.³–⁵ One of the most important differences between villous and tubular adenoma is the frequent concurrence of carcinoma in the former. The reported frequency varies from 30% to 70% of villous adenomas, depending on the size of the tumor.⁶–¹³

Here we report a case of advanced cancer coexisting with a very large villous adenoma of the rectum in a 54-year-old man without a family history of colorectal cancer. In an attempt to test the genetic model of adenoma-carcinoma progression, we analyzed alterations in the APC, k-ras, and p53 genes in samples obtained from subregions of the adenoma and carcinoma. While APC and k-ras mutations were detected in all samples from the adenoma and cancer, p53 mutation was detected only in the cancer. Based on these findings, we propose a likely sequence of genetic mutations which led to carcinogenesis in the present case.

Materials and Methods

Patient and Histopathology

The tumor was resected from a 54-year-old patient without a family history of colorectal cancer.
Mutations in a Large Villous Adenoma

Fig. 1. Resected specimen after fixation shows a large villous tumor of the rectosigmoid colon, 6.5 x 5.4 cm in size (a). Schematic drawing of the resected specimen (b). Horizontal stripes represent villous adenoma, stippled area moderately differentiated adenocarcinoma, and diagonally striped area ulceration. Sampling was done at N (normal), A1–3 (adenoma), and C1–3 (cancer).

It was located in the rectosigmoid colon and measured 6.5 x 5.4 cm. A type 2 advanced cancer was found in direct contact with the large villous tumor (Fig. 1a). The samples were dissected immediately after resection and processed for histopathological and subsequent genetic studies. The tumor was diagnosed as villous adenoma with moderate dysplasia, accompanied by moderately differentiated adenocarcinoma (Figs. 2 and 3) by three experienced gastrointestinal pathologists on the basis of the WHO histological typing of intestinal tumors.

DNA Extraction and PCR Amplification

Samples were collected from various subregions of the tumor and the adjacent normal mucosa (see Fig. 1b), fixed in 10% formalin and embedded in paraffin. Before DNA extraction, histological sections were screened for tumor cell enrichment. DNA was extracted from five 10-μm-thick sections. The sections were deparaffinized with xylene and then washed with 100% ethanol to remove the xylene, followed by treatment with proteinase, sodium dodecyl sulfate, and phenol-chloroform.

DNA samples were amplified for one cycle using the polymerase chain reaction (PCR) (5 min at 95°C once; 1 min at 95°C, 1 min at annealing temperature, 1 min at 72°C, for 40 cycles; and 7 min at 72°C). The annealing temperature for each reaction was 2–4°C below the Tm for the primer. The reaction mixture (100 μl) contained 200 ng of genomic DNA, 150 μM each primer, 200 μM each deoxynucleoside triphosphate, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 10% (w/v) glycerol, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Modified paired primers for exon 5-151 of Groden et al. (Table I) were used to amplify the 11 coding exons of the APC gene. The sequences of paired oligonucleotide primers for exon 1 of the k-ras gene and exons 5, 6, 7, and 8 of the p53 gene are listed in Table I.

Nonradioisotopic SSCP Analysis

In order to perform nonradioisotopic single-strand conformation polymorphism (SSCP) analysis, we modified the method described by Oto et al. Briefly, each PCR product was purified using chloroform and MicroSpin™ S-400 HR columns (Pharmacia P-L Biochemicals, Milwaukee, WI). Aliquots of the purified PCR products were mixed with equal volumes of formamide dye (95% formamide, 20 mM EDTA and 0.05% bromophenol blue and xylene cyanol). After denaturation at 80°C for 5 min, a 10-μl sample was immediately loaded on a neutral polyacrylamide gradient gel (10–20%). Two systems were run for each set of reactions: 25 mM Tris, 192 mM glycine, and 1 mM EDTA gel at 4°C, and 25 mM Tris, 192 mM glycine, 1 mM EDTA, and 5% glycerol gel at 21°C. Electrophoresis was carried out in a commercially available slab gel apparatus (138 x 130 x 0.75 mm, ATTO Co., Ltd., Tokyo). Electrophoresis was run at 30 W.
Fig. 3. Region of the tumor showing villous adenoma with moderate dysplasia (left) and moderately differentiated adenocarcinoma (right) in direct contact.

for 3 h for both systems using a temperature controller (Resolmax: ATTO Co., Ltd.). The gels were stained with a silver stain kit (Silver Stain Plus kit; BioRad Laboratories Co., Ltd., Hercules, CA).

Sequencing of Mutated Strands

Abnormal single-stranded DNA fragments were extracted with elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% SDS) from the corresponding bands on the SSCP gels by a modification of the "crush and soak" method. DNA fragments were amplified by asymmetric PCR in 100 μl of reaction mixture under the same conditions as those for PCR-SSCP analysis, except that the ratio of the forward to the reverse primers was 1:100. The amplified DNA samples were purified using a Millipore UFC3-THK column (Tokyo) for the dideoxy chain-termination reaction with 35S-dCTP using Sequenase, version 2.0 (United States Biochemical Co., Cleveland, OH). Primers used for sequencing were the same as those used in the PCR-SSCP.

Results

SSCP Analysis

Twenty pairs of primers were used to amplify DNA samples extracted from subregions (see Fig. 1) of the tumors. The PCR products comprising twenty independent sequences of 200–500 nt, each representing one of the exons 5–151 out of the thirty-eight coding regions of the APC gene, were subjected to SSCP analysis as described in Materials and Methods. The results are presented in Fig. 4. The PCR products for the APC exon 11 rev-

Fig. 2. Regions of the tumor showing villous adenoma with moderate dysplasia (a, b) and moderately differentiated adenocarcinoma (c).
MUTATIONS IN A LARGE VILLOUS ADENOMA

Table I. Sequences (5'-3') of Primers Used for PCR

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k-ras

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*p,* modified paired primers for exon 5-151 of the APC gene of Groden et al.13)

Sequencing

Abnormal single-stranded DNA fragments were extracted from the corresponding bands on SSCP gels. The DNA samples thus obtained from normal mucosa and two representative tumor sites, A1 and C3, were selected for sequencing of the APC and k-ras genes. Likewise, DNA samples from normal mucosa and two representative tumor sites, C1 and C3, were sequenced for the p53 gene (exon 7). For the APC gene, a TTG to TAG (stop codon) mutation was found at codon 479 in exon 11, resulting in premature termination. This was a novel mutation that has not been reported previously. The mutation of the k-ras gene, GGT to GAT, was located at codon 12 (exon 1) and that of the p53 gene, CGG to TGG, at codon 248 in exon 7 (Fig. 5a-c).

Discussion

Colorectal carcinomas are suitable for analyzing various stages of tumorigenesis, i.e., from very small adenomas to large metastatic carcinomas.12 A carcinoma in direct contact with an adenoma such, as seen in the present case, is an especially appropriate model for studying the assumed adenoma-carcinoma sequence, since it is possible that the carcinoma may have evolved from the adjacent adenoma.

In the genetic model of colorectal tumorigenesis, it is proposed that mutation of APC and possibly k-ras occurs early in adenoma formation, while that of p53 is a late event.12 Germine mutations of the APC gene have been found to be responsible for the inherited predisposition to familial adenomatous polyposis (FAP).13 Moreover, the APC gene is somatically inactivated by mutations...
and/or LOH in the majority of sporadic colon
tumors. Over 95% of such mutations are
predicted to result in premature truncation of the
APC protein. In the present case, it was shown
that the villous adenoma and its adjacent cancer
contained the same mutation, TTG to TAG, at
codon 479 in exon 11 of the APC gene. Although
a newly created stop codon was previously report-
red for this gene, the mutation at codon 479 in exon 11
found in the present study appeared to be
a novel one. It is also known that the frequency of
APC mutations in colorectal adenomas is signifi-
cantly higher in lesions with a conspicuous villous
morphology than in those without it.

Another genetic change that is assumed to follow
APC mutation in the sequence of colorectal tumori-
genesis is k-ras mutation. Approximately 50% of
both colorectal carcinomas and adenomas greater
than 1 cm in diameter have been found to have a
ras mutation. The frequency of k-ras mutation is
known to increase with development from moderate
to severe dysplasia and with increased adenoma
size. It is also known that the type of k-ras point
mutation is correlated with the degree of malignan-
cy of colorectal cancer, although k-ras mutation
alone has no direct effect on the rate of progres-
sion of adenoma to carcinoma, at least in
FAP. The present finding that both adenoma
and carcinoma possessed the same type of k-ras
mutation suggested two possibilities: the carcinoma
evolved from the adenoma adjacent to it, or the
two adjacent but independent tumors happened to
undergo the same mutation at codon 12 of the k-
ras gene.

Another genetic event contributing to the
adenoma-carcinoma sequence is inactivation of p53,
a tumor suppressor gene. Genetic changes in the
p53 gene are not always detected in colon adeno-
MUTATIONS IN A LARGE VILLOUS ADENOMA

mas, but are frequently evident in advanced colon carcinomas and are a constant feature of almost all metastatic carcinomas. It has been suggested that genetic changes in the p53 gene are involved in the conversion of adenoma to early carcinoma.26–28 More than 90% of p53 gene mutations detected in colorectal tumors are missense point mutations, in contrast to the amber mutations seen in APC. The mutations are clustered in exons 5–8, domains that have been evolutionarily conserved for DNA-binding. In the present study, only the carcinoma and not the adenoma had a missense point mutation, CGG(Arg) to TGG(Trp), at codon 248 in exon 7, a mutational hotspot of the p53 gene.

It is a matter of controversy whether a human neoplasm showing intratumoral heterogeneity has a monoclonal or polyclonal composition. Recently, several investigators have tried to provide objective evidence for tumor clonality. However, their studies were based on analysis of only a single genetic change, or at most two.29,30 It is probably more convincing to assess tumor clonality by identifying multiple genetic changes, as was done in the present study.

There has been an earlier report indicating that while APC mutation was distributed homogeneously throughout adenomas, synchronous APC and k-ras mutations were detected only in small discrete areas.31 This finding suggests that k-ras mutation was acquired after APC mutation, and that tumor heterogeneity must have been present until clonal dominance was reestablished. Based on the current hypothesis of colorectal tumor progression, the present study appears to support the possibility that both the adenoma and carcinoma observed in this case originated from a single cell, and that mutations of APC and k-ras played significant roles in the formation and development of villous adenoma, while an additional mutation of p53 contributed to progression of the villous adenoma into cancer.

Acknowledgments

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References

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