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Somatic Mutations of the *APC* Gene in Primary Breast Cancers

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APC* gene mutations play an important role in the initiation step of colorectal carcinogenesis in both familial adenomatous polyposis (FAP) patients and non-FAP patients. Although the *APC* gene is expressed in most tissues, including the lung, liver, kidney, and mammary gland, its somatic mutations have rarely been found in primary tumors affecting these organs. We have developed a sensitive yeast-based assay for screening almost the entire coding region of the *APC* gene. By this method, we have been able to detect somatic mutations of the *APC* gene in 57% of colorectal cancers and none in non-small cell lung cancers. Interestingly, the assay detected somatic *APC* gene mutations in 18% of breast cancers, in which *APC* gene mutation was previously considered rare. In the breast cancers, most of the *APC* mutations were distributed outside the mutation cluster region that has been advocated for colorectal cancers. We also noted a difference in the mutation pattern of the *APC* between colorectal and breast cancers. In colorectal cancers, all base substitutions were observed at C residues (5 of 5), whereas in breast cancers the majority of them were found at G residues (4 of 5). Furthermore, *APC* mutations were observed at a significantly high frequency in advanced stages of primary breast cancers (TNM classification, $P < 0.05$; T category, $P < 0.01$). Our data suggest that the etiology of the *APC* mutations and their biological role in carcinogenesis may differ between colorectal and breast cancers. *(Am J Pathol 2000, 156:1997–2005)

Mutations of the *APC* tumor-suppressor gene are responsible for both familial and sporadic colorectal cancers.[1,2] Somatic mutations of this gene are also implicated in extracolonic cancers such as those of the pancreas,[3] stomach,[4] and esophagus.[5] However, only a few *APC* mutations have been reported in human primary breast cancers to date, in contrast to those in gastrointestinal tract cancers,[6] although it is known that female mice carrying *Min*, a nonsense mutation at codon 850 of the *Apc*, occasionally develop mammary carcinomas.[7]

The *APC* gene at chromosome 5q21 is transcribed into nearly 9.5 kb-sized mRNA which encodes a huge protein of 2,843 amino acids.[2,8] The coding region consists of 15 exons, the last containing more than three quarters of the whole coding region. In sporadic colorectal cancers, >60% of the somatic mutations are clustered within a small region of exon 15 designated as the mutation cluster region (MCR), which accounts for <10% of the coding region. However, most familial adenomatous polyposis (FAP) patients carry truncation mutations in the N-terminal half.[9] Hence, the widespread distribution of many different mutations within the very large *APC* gene poses problems in molecular genetic diagnosis, and detection of the *APC* mutations is labor-intensive when nucleic acid-based approaches are used. An alternative method is the protein truncation test which detects the protein truncating mutations *in vitro*[10,11] or in yeast,[12] because the vast majority (>95%) of the *APC* mutations have truncation of the APC protein because of frameshift, nonsense, or splice-site mutations.[13]

We present here a new, rapid, and sensitive yeast-based screening method for detection of the truncating mutation in the *APC*. The assay has advantages over the similar one which uses *URA3* selection,[14] because it does not require a replica-plating step and can easily distinguish yeast containing mutant *APC* as red colonies. Moreover, mutant *APC* plasmid can be easily recovered from red colonies and subjected to DNA sequence analysis. The assay therefore enables us to easily test a large number of samples and many clones per sample. We applied this assay to analyze the *APC* mutations in clinical samples of colorectal, breast, and lung cancers.

Materials and Methods

Cell Lines and Clinical Samples

Six human colon cancer cell lines (DLD1, Colo201, Colo320DM, HT29, HCT116, and SW480) were obtained

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from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer at Tohoku University. All cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% fetal calf serum and 0.03% L-glutamine. Resected specimens of normal colonic mucosa, two polyps, 23 colorectal cancers, 70 breast cancers, and 29 non-small-cell lung carcinomas were obtained from the First Department of Surgery, Hokkaido University Hospital, Japan. They were snap-frozen in liquid nitrogen and stored at −80°C until analyzed. No procedure to enrich the samples for tumor cells was carried out in this study. The clinical stage classifications of the colorectal and breast cancers were based on the TNM classification of the International Union Against Cancer. Breast cancers were histologically typed according to the "General Rules for Clinical and Pathological Recording of Breast Cancer" issued by the Japanese Cancer Society.

RNA and DNA Preparation

Total RNA was extracted from cell lines and tumor tissues. Approximately 100 mg of tumor samples or 1×10^5 cells were homogenized in 2 ml of Trizol reagent (Life Technologies, Inc., Tokyo, Japan), mixed with 0.4 ml of chloroform, and centrifuged. The supernatant was collected and total RNA was precipitated with isopropyl alcohol. The precipitate was washed with 75% ethanol and dissolved with 40 μl of water containing 0.1% diethyl pyrocarbonate. RNA quality was verified by electrophoresis in a 1.0% agarose gel. cDNA was synthesized at 37°C for 1 hour with 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) from 3 μg of total RNA by using a random pdN6 primer (Life Technologies, Inc.). Genomic DNA was extracted from cells and tissues or from the residue in RNA extraction.

Polymerase Chain Reactions (PCRs)

Regions I and II (see Figure 3) of the *APC* coding sequence were amplified from mRNA-derived cDNA whereas regions III to V were amplified from genomic DNA. All PCR fragments was obtained by using proofreading *Pfu* DNA polymerase (Stratagene, La Jolla, CA), which can minimize base misincorporation. Primers for amplification of the five regions were 5′-ACGGCCGCAAAAAA TGGCTGCAGCTTCATATGAT-3′ and 5′-TCGGCCGCCCCCCTCCCAA TAATTCTGCAATGGCCTGTAG-3′ for region I; 5′-TCGGCCGCAAAA AATGCGCGCTTACTGTGAAACCTG-3′ and 5′-TCGGCCGCCCACCTCCCCTTCCAGAGTTCAACTGCTCATC-3′ for region II; 5′-AAAAGCTTAAATGAAACCCTCGATTGAATCC-3′ and 5′-CGGCCGCTGCTCTGATTCTGTTTCATTCCC-3′ for region III; 5′-TCGGCCGCAAAAAATGGCCACGGAAAGTACTCCAGATG-3′ and 5′-TCGGCCGTTCCAATGTACTTTTCTCCCCTGG-3′ for region IV; 5′-TCGGCCGAAAATGGATTGGAAAGCTATTCAGGAAG-3′ and 5′-ACGGCCGCCCAACAGATGTCACAAGGTAAGACC-3′ for region V. The PCR conditions are available from the authors on request.

Plasmid Construction

The *ADE2* expression vector pLF-ADE2 was constructed as follows. A fragment containing a *Saccharomyces cerevisiae ADE2* coding sequence from the initiation codon to the natural termination codon was amplified by PCR using primers that contained a unique *Not*I site at the 5′ terminus. The primers were 5′-AGCGGCCGCGTATGGATTCTAGAACAGTTGG-3′ and 5′- CTCGGCCGTTACTTGTTTTCTAGATAAGCTTC-3′. The plasmid pLS76[15] was digested with *Hin*dIII and *Eag*I, blunt-ended by T4 DNA polymerase, and ligated with the *ADE2* fragment. In this process, a unique *Not*I site was generated on the 5′ end of the fragment, thereby facilitating the subsequent insertion of five fragments of the *APC* cDNA. Five overlapping fragments spanning codons 1 to 497, codons 413 to 1040, codons 979 to 1541, codons 1492 to 2177, and codons 2100 to 2843 of the *APC* cDNA were amplified and inserted in-frame into the *Not*I site of the pLF-ADE2 to produce pLF-ADE2-I, -II, -III, -IV, and -V, respectively. Some gap vectors were made by replacing the central portions of the inserted fragments with unique restriction sites: *Bgl*II (pLF-I-ADE2g), *Bgl*II and *Nde*I (pLF-II-ADE2g), *Nde*I and *Pst*I (pLF-III-ADE2g), *Pst*I and *Bcl*I (pLF-IV-ADE2g), and *Bcl*I (pLF-V-ADE2g). The pLF-ADE2-III was digested with *Pst*I to delete codons 1337 to 1475 of *APC* cDNA and self-ligated to create an out-of-frame deletion mutant (pLF-ADE2-IIImt). The other mutant clone (pLF-ADE2-IVmt) was prepared by inserting a single adenine at codon 2050 using PCR mutagenesis. These mutant clones were used for the assessment of the yeast color assay system.

APC Yeast Color Assay

The yeast strain yIG397 (genotype; MATa *ade2-1 lue2-3, 112trp1-1 his3-11, 15 can1-100*) was used throughout this study. When the strain was cultured on minimal medium plus adenine (5 μg/ml), the cell formed red colonies because of the accumulation of an intermediate in adenine metabolism.[16] The yeast was cultured in 100 ml of yeast extract/peptone/dextrose medium supplement with 200 μg/ml of adenine, until OD_{600} reached 0.8. The cells were collected, washed, and resuspended in an equal volume of LiOAc solution (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 0.1 mol/L lithium acetate). For each transformation, 50 μl of yeast suspension was mixed with 1 to 5 μl of unpurified *APC* cDNA PCR products, 25 to 150 ng of each linearized plasmid, 50 μg of sonicated single-stranded salmon sperm DNA, and 300 μl of LiOAc solution containing 40% polyethylene glycol 4000 (Kanto Chemical, Tokyo, Japan). The mixture was incubated at 30°C for 30 minutes and heat-shocked at 42°C for 15 minutes. Yeast transformants were pelleted and plated on a synthetic minimal medium minus leucine plus adenine (5 μg/ml) and incubated for 48 hours in a 30°C humidified atmosphere. These are summarized in Figure 1. More than 200 colonies were examined in this assay. When the percentage of red colonies was 10 to 20%, the assay was repeated and the mean value was presented.

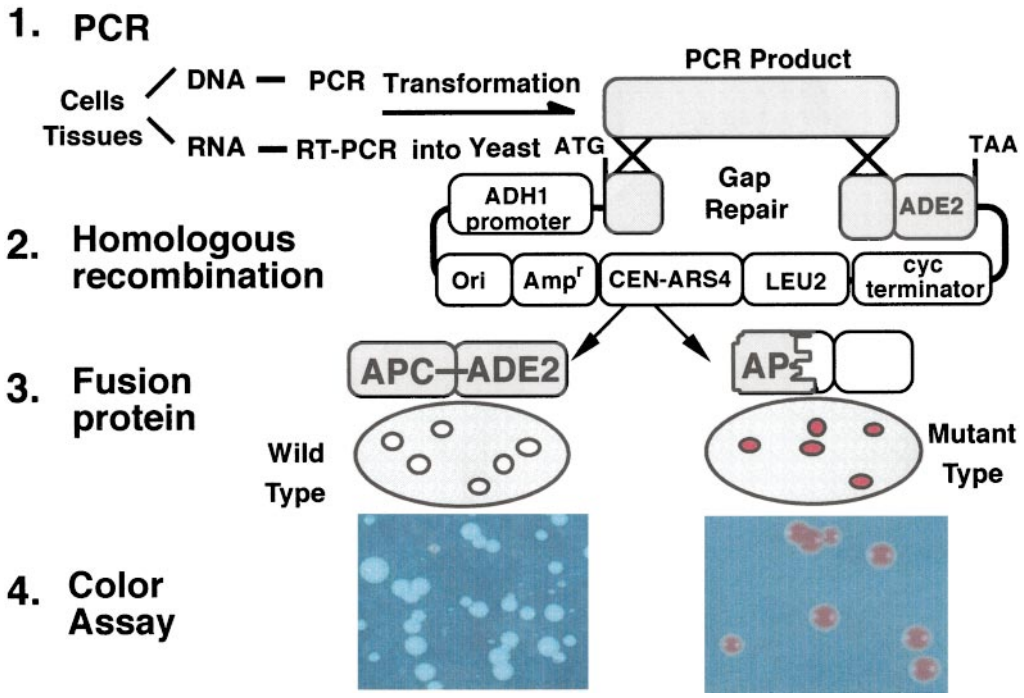

Figure 1. Schematic representation of yeast color assay. Step 1 (PCR): *APC* mRNA (region I–II) and genomic DNA (region III–V) were amplified by PCR and co-transformed into yeast with a linearized expression vector carrying the 5′ and 3′ ends of the *APC* cDNA. Step 2 (homologous recombination) and Step 3 (fusion protein expression): Gap repair of the plasmid with the PCR product resulted in constitutive expression of APC-ADE2 fusion protein, driven from the ADH1 promoter. Yeast transformants that contained gap-repaired plasmid were selected on a medium lacking leucine. Step 4 (color assay): Although the medium contained sufficient adenine for the growth of ADE2$^-$ cells, they formed red colonies. Yeast containing cDNA (or DNA) fragment of wild-type *APC* and of mutant (truncating) *APC* formed white and red colonies, respectively.

Correlation between Mutant APC Contents and Percentage of Red Colonies

The HCT116 (wild-type *APC*) and DLD1 cells (mutant *APC*) were mixed at serial ratios, 0, 5, 10, 15, 20, 25, 30, 50, 75, and 100% (DLD1 cells/total cells). They were equally divided into two and cultured for 6 hours, and then total RNA and genomic DNA were extracted. Region III was chosen as a representative of the five regions, and RNA and genomic DNA were used as templates for reverse transcriptase-PCR and PCR amplification, respectively. The PCR products were subjected to the yeast color assay repeatedly and the mean value was presented.

Recovery of Plasmid from Yeast and DNA Sequencing

Yeast was digested with zymolyase 100T (Seikagaku-Kogyo, Tokyo, Japan), and plasmids were recovered by an alkaline lysis method (QIAprep plasmid kit, Qiagen, Hilden, Germany) and transfected into an *Escherichia coli* XL1Blue strain by electroporation. Recovered plasmids were subjected to sequence analysis on an ABI 377A automated sequencer (Applied Biosynthesis, Urayasu, Japan) using Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Life Science, Tokyo, Japan) and appropriate *APC*-specific oligonucleotide primers. To identify clonal mutations in mutation-positive cases, plasmids rescued from three to 10 colonies were sequenced and the presence of identical mutation was confirmed.

Statistical Analysis

The relationship between ratios of cells (DLD1) with *APC* mutation and the percentage of red colonies was studied by a regression analysis. Correlations of the clinical parameters of the patients and their *APC* status were analyzed by a Yates-corrected chi-square test.

Results

Development of APC Yeast Color Assay

We divided the wild-type *APC* coding sequence of 8.5 kb into five overlapping parts, and each corresponding PCR-amplified fragment was ligated in-frame with an *ADE2* open reading frame. The resulting constructs, pLF-ADE2-I, -II, -III, -IV, and -V, preserved the ADE2$^+$ phenotype (white yeast colony) after the introduction into the yeast (Figure 1). On the other hand, when plasmids containing frameshift mutations, pLF-ADE2-IIImt and pLF-ADE2-IVmt, were transfected into yeast, the yeast showed an ADE2$^-$ phenotype (red yeast colony) because of the accumulation of an intermediate in the adenine metabolism (Figure 1).[16] Thus, the *APC-ADE2* reporter plasmids correctly discriminated in-frame and out-of-frame *APC* sequences. For the yeast color assay of clinical samples, we prepared linearized gap vectors,

Table 1. Summarized Results of *APC* Yeast Color Assay and Subsequent Sequencing of Colon Cancer Cell Lines

Cell lines	% Red colonies				
	Region I	Region II	Region III	Region IV	Region V
HCT116	5.8	6.4	6.3	0.0	4.1
DLD1	8.5	7.5	97.7 (1414:GGC→GG)	7.6	51.3 (2166:CGA→TGA)
SW480	0.0	1.6	99.3 (1338:CAG→TAG)	7.2	5.2
Colo201	0.0	5.6	4.9	97.3 (1554:GAA→GAAA)	5.7
Colo320DM	8.1	95.5 (811:TCA→TGA)	7.6	5.6	6.4
HT29	7.8	28.8 (853:GAG→TAG)	4.2	74.5 (1554:GAA→GAAA)	3.9

pLF-ADE2-Ig, -IIg, -IIIg, -IVg, and -Vg, by removing most of the *APC* inserts, leaving ~60–150 bp of the flanking *APC* sequences to allow homologous recombination. Because the *APC* expression vectors are linearized at codons 20 and 446 (region I), codons 446 and 1,013 (region II), codons 1,013 and 1,516 (region III), codons 1,516 and 2,153 (region IV), and codons 2,153 and 2,808 (region V), the assay can test almost the entire *APC* coding sequence (see Figure 3). To test the fidelity of the system, we amplified region III of human *APC* cDNA with *Pfu* DNA polymerase. We cloned an unpurified PCR product into the constitutive yeast expression vector pLF-ADE2-IIIg by homologous recombination *in vivo* after co-transfection of the PCR product and linearized gap vector into yeast, followed by selection of recombinants in plates lacking leucine, as described previously.[17] PCR-amplified wild-type and mutant cDNA gave 2.9 ± 0.5% and 100% of red colonies, respectively.

Testing the Assay with Colon Cancer Cell Lines

We examined five human colon cancer cell lines (four with mutant *APC* and one with wild-type *APC*) to assess the validity of the yeast color assay (Table 1). Total RNA and genomic DNA were extracted from these cell lines. Each region of *APC* cDNA consisted of multiple exons or a single exon; ie, region I (exons 1 to 10), region II (exons 10 to 15), and regions III to V (exon 15). We therefore used RNA for the assay of regions I and II, and genomic DNA for the assay of regions III to V. More than 96% red colonies were observed in one of the five regions when the assay was performed in SW480, Colo201, and Colo320DM cells. A sequencing analysis demonstrated mutations at codon 1,338 (CAG to TAG) in SW480, 1,554 (GAA to GAAA) in Colo201, and 811 (TCA to TGA) in Colo320DM. The DLD1 cell gave ~100% and 50% red colonies in regions III (mutation at codon 1,414, GGC to GG) and V (codon 2,166, CGA to TGA), respectively. HT-29 cells gave 30% and 70% red colonies in regions II (codon 853, GAG to TAG) and IV (codon 1,556, ACT to AACT), respectively. HCT116 cells with wild-type *APC* gave low percentages of red colonies (0 to 6.4%) in all of the regions. Because normal colonic mucosa gave 2 to 9.9% red colonies (Table 2), the higher values were thought indicative of the presence of *APC* mutations.

Correlation between Mutant APC Contents and Percentage of Red Colonies

DLD1 and HCT116 cells have been shown to express similar levels of APC protein.[18] To assess the linearity of the assay, we mixed HCT116 (wild-type *APC*) and DLD1 cells (mutant *APC)* at serial ratios and subjected them to the assay. We chose region III as a representative of the five regions, and used RNA and genomic DNA as templates for reverse transcriptase-PCR and PCR amplification, respectively. As shown in Figure 2, a highly linear input-output relation was observed in both DNA ($r = 0.99$) and RNA ($r = 0.99$) samples. RNA and DNA samples obtained from identical cell mixtures gave almost the same percentage of red colonies, which indicated that nonsense-mediated RNA decay was negligible in this assay.

Testing the Assay with Samples from a FAP Patient

We used the new assay to test polyps (polyp-1 and -2) from a FAP patient. The polyp-1 gave red colonies of 50% in region III (Table 2), and sequencing of plasmids rescued from multiple red colonies showed that the patient was heterozygous for mutant *APC* allele (Q1114X). Polyp-2 gave red colonies of 79% in region III and the sequencing analysis revealed that a new somatic mutation at codon 1,309 was introduced into normal APC allele.

Identification of APC Mutations in Colon Cancers

To test the suitability of the *APC* yeast color assay for analyzing tumor samples, RNA and DNA were extracted from sporadic colorectal cancers, and subjected to the assay. The results were summarized in Table 2. Out of 23 cancers tested, 13 cancers (57%) gave red colonies of >10%. Sequence analysis revealed truncating *APC* mutations in these samples; 10 cancers with one mutation and three with two mutations. Clonality was demonstrated by sequencing plasmids rescued from multiple red col-

Table 2. APC Mutations* Detected by *APC* Yeast Color Assay in Polyps from a FAP Patient and Sporadic Colorectal Carcinomas

Samples	Histology§	TNM	% Red colonies DNA	% Red colonies RNA	Region	Codon	Mutation	Clonality DNA	Clonality RNA
Mucosa	W	III	2.0 ~ 9.9		I~V				
Polyp† 1			ND	50	III	1114	CGA to TGA	ND	4/4
Polyp† 2			ND	79	III	1114	CGA to TGA	ND	6/10
Polyp† 2				79	III	1309	GAAAAGA to GA	ND	4/10
C1	W	0	19	17	II	876	CGA to TGA	4/4	4/4
C2‡	W	0	50	50	II	799	TAT to T	3/3	4/4
C2‡			30	33	IV	1554	GAA to GAAA	3/3	3/4
C3	W	I	32	28	III	1436	AGT to AG	4/4	4/4
C4	M	I	20	22	III	1396	TTT to TTTTT	3/4	4/4
C5	W	I	49	48	III	1196	TCA to TAA	3/3	4/4
C5			23	25	IV	1790	AGA to AGAA	3/3	2/4
C7	W	II	51	57	III	1450	CGA to TGA	3/3	3/3
C9	W	III	45	52	IV	1554	GAA to GAAA	4/5	4/4
C10	W	III	29	33	III	1114	CGA to TGA	4/4	3/3
C10			17	20	IV	1545	TCA to TAA	3/3	3/4
C14	M	III	34	39	III	1462	AAG to A	4/4	4/4
C15	M	III	33	30	III	1309	GAAAAGA to GA	4/4	3/3
C16	M	III	37	39	III	1319	CCT to CT	3/3	4/4
C21	P	IV	24	26	IV	1628	AAG to AG	2/4	3/4
C23	M	IV	32	ND	III	1462	AAG to A	4/4	ND

*Cases without APC mutation are not shown.
†Two polyps of an FAP patient.
‡C2, another FAP patient.
§Histology: W, well; M, moderate; P, poor; ND, not done (absence of DNA or RNA samples).

onies given by both RNA and DNA samples. The RNA and DNA samples from the identical cancers gave almost the same results with respect to the percentage of red colonies and clonality of the mutations. Ten out of 16 somatic mutations (62%) were found in the *APC* mutation database[19] and eight out of the 16 somatic mutations (50%) were located in the mutation cluster region (MCR, codons 1,296 to 1,513) (Figure 3). Of five nonsense mutations identified, three were C to T and two were C to A base substitution. All of the insertion mutations were found in mononucleotide repeats of adenine or thymine. The mutations were found more frequently in TNM stages 0 to I (5 of 5) than stages II to IV (8 of 18). Similarly, the majority of the mutations (7 of 10) were found in well-differentiated adenocarcinomas.

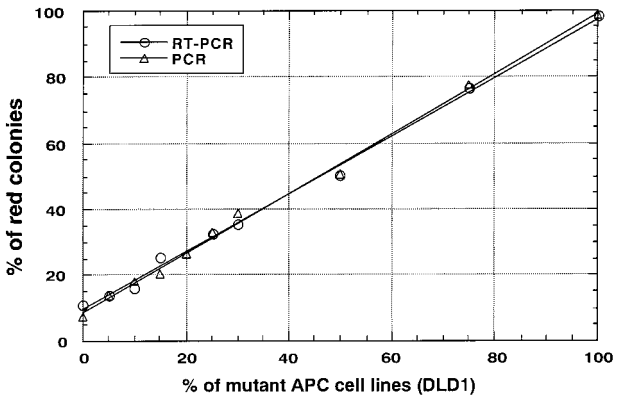

Figure 2. Correlation between mutant *APC* contents and percentage of red colonies in the yeast color assay. A positive correlation was observed between the contents of mutant *APC* and percentage of red colonies, which was evaluated by simple regression based on the following formula. $y = 4.67 + 0.94x$; $r = 0.998$ (genomic DNA), $y = 5.23 + 0.93x$; $r = 0.998$ (RNA). △, Genomic DNA samples; ○, RNA samples.

Identification of APC Mutations in Breast Cancers

We screened 70 sporadic breast cancers by the assay and the results are summarized in Table 3. Out of 70 RNA samples from breast cancers, 11 samples gave >15% red colonies in a single region (I to V) of *APC* cDNA and two samples gave those in double regions. DNA sequencing demonstrated the clonality in all these samples (Table 3). These mutations were confirmed by the yeast color assay on the corresponding genomic DNA regions. However, in the four regions, B40 (region III), B42 (IV), B44 (IV), and B59 (IV), which gave low percentages of 11 to 12% red colonies and low clonality (2 of 4 or 2 of 6) of mutations, sequencing of the corresponding genomic DNA regions failed to show identical clonal mutations. Consequently, clonal *APC* gene mutations were confirmed in 13 of 70 (18%) breast cancers (Table 3). Two tumors had heterozygous mutations. Among the total of 15 mutations identified, two were identical (samples B41 and B50, codon 1795 TCA→TCAA); ie, 14 different mutations were identified.

Unlike those seen in colorectal cancers, mutations found in breast cancers were widely distributed between codons 1058 and 1795 of the *APC* coding region and only 4 of 15 (27%) were located in the MCR (Figure 3). Furthermore, the majority of base substitutions were G to T (4 of 5) and approximately half of the mutations (8 of 14, 57%) were not found in the *APC* mutation database.[19] Of 10 frameshift mutations, 50% were single-base insertions and 50% were single-base deletions. All of the insertion mutations were found in the sites of six runs of adenine residues. In regard to histological typing of the tumors, the majority of the mutations were found in solid tubular

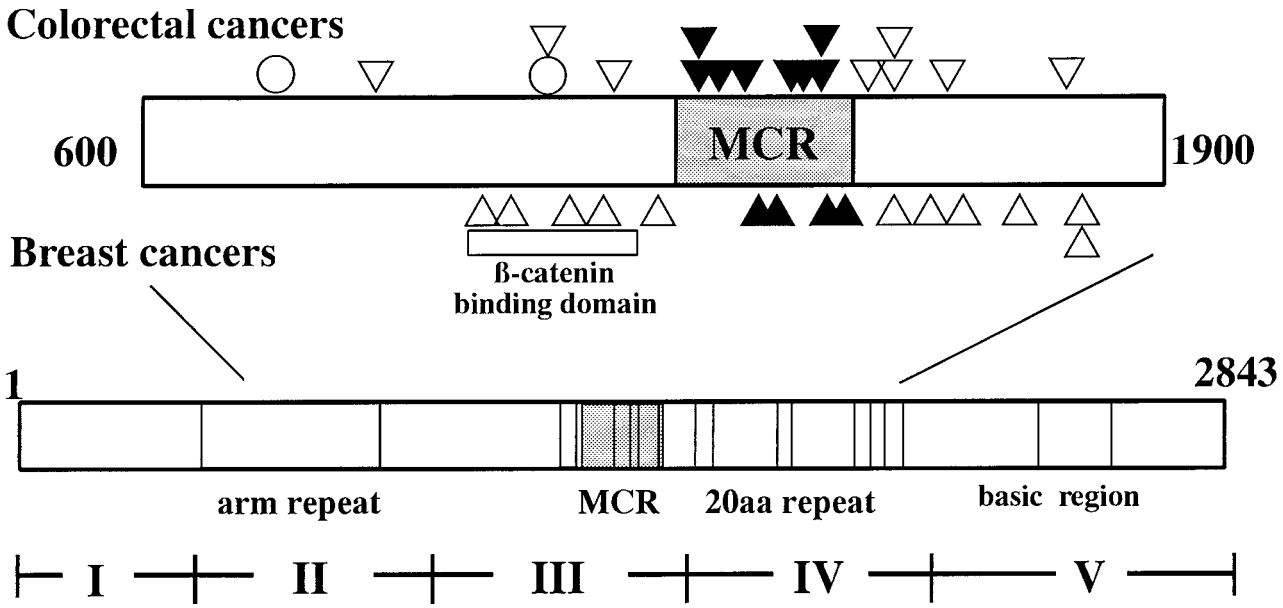

Figure 3. The distribution of *APC* mutations in colorectal and breast cancers. The mutations listed in Tables 2 and 3 are shown above (colorectal cancers) and below (breast cancers) the **rectangle box** (codons 600 to 1900) (**top**). The second structure of wild-type APC protein and the regions examined by the yeast color assay are shown at the **bottom**. □, germline mutation in FAP patients; ▽, somatic mutation in colorectal cancer; △, somatic mutation in breast cancer; ▲▼, mutations located at the mutation cluster region (codons 1,296 to 1,513) reported in colorectal cancers.[9]

carcinomas (7 of 25) or papillotubular carcinomas (5 of 27), whereas only one was detected in scirrhous-type tumors (1 of 14). Histological grading of the detected mutations were grade 1, grade 2, and grade 3 in 1 of 18 (6%), 6 of 32 (19%), and 6 of 20 (30%) samples, respectively. The mutation rate of the *APC* gene was significantly higher in stages 3 and 4 (5 of 10, 50%) than in stages 1 and 2 (8 of 60, 13%; $P < 0.05$). Consequently, *APC* mutations were frequently found at the high-grade and advanced stage of primary breast cancers. A highly significant association was also observed between the *APC* mutation and the size of primary tumor (T category): 8 of 62 (13%) in T1 and 2 *versus* 5 of 8 (63%) in T3 and 4 ($P < 0.01$) (Table 3).

We also screened 29 non-small-cell lung cancers (21 adenocarcinomas and eight squamous cell carcinomas) by the *APC* yeast color assay. All of the cancers gave background level (<10%) of red colonies and were scored as negative for the *APC* mutation. We confirmed no clonal mutation of the *APC* gene in the samples which gave around 10% red colonies; ie, no *APC* mutations were detected in the 29 lung cancers.

Discussion

Mutations of the *APC* tumor suppressor gene have been found in cancers of the digestive tract system including colorectal, gastric, esophageal, and pancreatic cancers, but reported to be quite rare in breast cancers.[6] In the present study, we demonstrated truncating-type mutations of the *APC* gene in up to 18% of primary breast cancers, and showed that the *APC* gene mutation is far more frequent in human breast cancers than previously thought. To our knowledge, *APC* mutations have not been demonstrated in breast cancers at such a high rate, whereas several studies indirectly suggested it, eg, 40% LOH within *APC* gene[20,21] and reduced or lost expression (40.7%) of APC protein in breast cancers.[22] The difference in the frequency of the *APC* mutation between in the present study and previous ones may be explained by the difference in the efficiency of screening methods used for detection of the *APC* mutation. The single-strand conformation polymorphism analysis, the most frequently used method in the mutational studies of the *APC* gene, is rather labor-intensive and suffers from considerable variation among laboratories,[1] resulting in variable sensitivity which even in the best hands rarely permits detection of all mutations.[17] Moreover, the large size of the *APC* gene has often hampered the previous mutation studies. The protein truncation test originally developed for detection of *APC* gene mutations needs laborious cloning of *APC* cDNA in several parts.[11] This test is susceptible to contamination of a large amount of normal cells. Moreover, truncation of APC protein at the beginning or end of translation is often overlooked at the Western blotting step because of the low translational efficiency or limited electrophoretic resolution. The present *APC* yeast color assay, in this regard, is considerably more efficient and sensitive than the previous method. It is conceivable that this assay may have introduced some artifactual bias, but we consider this unlikely; because of the agreements in the results of mRNA and genomic DNA. Detection of no *APC* mutation in lung cancers, in contrast to the high detection rate in colonic cancers, also indicates a very low false-positivity in this assay.

Although a similar yeast-based assay has been reported for detection of the *APC* gene mutation, the advantages of the present yeast color assay, compared

Table 3. Summarized Results of *APC* Yeast Color Assay and Subsequent Sequencing of Breast Cancers

Samples	Age	Histological classification*	T†	Grade	Stage	% Red colonies (Region)	Codon	Mutation	Clonality RNA	Clonality DNA
B1	44	Solid-tub	3	2	3a	23 (IV)	1591	GCA to GCAA	4/5	ND‡
B2	46	Pap-tub	2	3	2	6 ~ 7				
B3	42	Solid-tub	3	3	3a	27 (III)	1106	GGA to TGA	3/3	5/5
B4	46	Pap-tub	2	2	4	5 ~ 6			0/8	
B5	47	Scirrhous	2	2	2	3 ~ 5				
B6	52	Scirrhous	2	3	2	5 ~ 7				
B7	64	Pap-tub	2	2	2	4 ~ 9				
B8	54	Solid-tub	2	3	2	6 ~ 7				
B9	65	Pap-tub	2	2	2	6 ~ 9				
B10	40	Solid-tub	2	2	2	7 ~ 10			0/4	
B11	26	Pap-tub	1	2	1	3 ~ 7				
B12	53	Pap-tub	1	2	1	15 (III)	1250	AAA to AA	5/8	ND
B13	45	Pap-tub	2	2	2	5 ~ 8				
B14	56	Scirrhous	2	3	3a	5 ~ 8				
B15	71	Pap-tub	2	1	2	6 ~ 9				
B16	45	Pap-tub	2	1	2	2 ~ 9				
B17	68	Scirrhous	2	3	2	19 (III)	1058	GAT to GA	3/4	ND
B18	60	Pap-tub	2	3	2	3 ~ 4				
B19	41	Solid-tub	1	3	2	4 ~ 8				
B20	52	Solid-tub	2	1	2	6 ~ 8				
B21	54	Scirrhous	3	2	4	4 ~ 5				
B22	42	Solid-tub	2	3	2	6 ~ 8				
B23	32	Scirrhous	2	2	2	4 ~ 9			0/8	
B24	45	Pap-tub	3	3	3a	48 (III)	1408	GAA to AA	3/4	ND
B25	58	Solid-tub	4	3	3b	31 (III)	1173	GTG to GT	4/5	ND
B25						35 (IV)	1683	GAA to TAA	4/4	ND
B26	51	Scirrhous	2	3	2	4 ~ 5				
B27	35	Noninvasive	2	2	2	3 ~ 6				
B28	33	Pap-tub	2	2	2	4 ~ 9				
B29	47	Solid-tub	2	1	2	2 ~ 6				
B30	64	Special type	2	1	2	3 ~ 9				
B31	71	Scirrhous	2	1	2	7 ~ 9				
B32	58	Solid-tub	1	2	1	5 ~ 7				
B33	38	Solid-tub	2	1	2	5 ~ 6				
B34	48	Special type	1	1	1	5 ~ 7				
B35	61	Pap-tub	2	1	2	6 ~ 8				
B36	69	Solid-tub	2	2	2	7 ~ 10			0/4	
B37	43	Pap-tub	2	1	2	0 ~ 8				
B38	61	Solid-tub	2	1	2	2 ~ 5				
B39	44	Solid-tub	1	2	1	4 ~ 10			0/5	
B40	63	Solid-tub	1	3	1	11 (III)			2/4	0/4
B41	51	Pap-tub	2	1	2	19 (IV)	1795	TCA to TCAA	3/3	3/3
B42	49	Solid-tub	1	2	1	22 (III)	1464	GAG to TAG	4/4	4/4
B42						12 (IV)			2/4	0/4
B43	55	Solid-tub	2	2	2	2 ~ 9			0/4	
B44	62	Pap-tub	2	3	2	11 (IV)			2/6	0/4
B45	69	Scirrhous	1	2	2	8 ~ 10			0/4	
B46	58	Pap-tub	2	3	2	4 ~ 10			0/4	
B47	49	Pap-tub	2	2	2	5 ~ 9				
B48	76	Scirrhous	4	1	3b	2 ~ 9				
B49	46	Solid-tub	2	2	2	4 ~ 9				
B50	58	Solid-tub	2	2	2	56 (IV)	1795	TCA to TCAA	5/7	3/3
B51	44	Pap-tub	1	1	1	6 ~ 8				
B52	61	Pap-tub	1	2	1	5 ~ 7				
B53	51	Solid-tub	2	1	2	6 ~ 9			0/6	
B54	49	Pap-tub	2	2	2	7 ~ 8				
B55	59	Solid-tub	2	2	2	5 ~ 7				
B56	45	Scirrhous	2	2	2	3 ~ 8				
B57	60	Special type	2	3	2	7 ~ 10			0/4	
B58	43	Pap-tub	2	1	2	3 ~ 8				
B59	57	Pap-tub	2	2	2	16 (III)	1191	CAG to TAG	3/4	3/4
B59						11 (IV)			2/4	0/4
B60	70	Solid-tub	4	3	3b	20 (III)	1499	GGA to TGA	4/6	3/3
B61	83	Solid-tub	2	1	2	7 ~ 10			0/4	
B62	49	Solid-tub	1	3	1	4 ~ 7				
B63	45	Pap-tub	2	3	2	4 ~ 8				
B64	48	Pap-tub	2	2	2	28 (IV)	1703	GGA to GGAA	4/6	ND
B65	57	Pap-tub	4	2	4	3 ~ 10			0/10	
B66	60	Pap-tub	2	2	2	6 ~ 10			0/7	
B67	62	Scirrhous	1	2	1	6 ~ 8				
B68	50	Scirrhous	2	1	2	4 ~ 7				
B69	36	Solid-tub	1	3	1	30 (III)	1413	ATG to AT	3/4	4/4
B69						23 (IV)	1554	GAA to GAAA	3/5	4/4
B70	45	Scirrhous	2	2	2	7 ~ 8				

*Pap-tub, Papillotubular; solid-tub, solid-tubular; noninvasive, noninvasive ductal.
†T, primary T category.
‡ND, Not done (absence of DNA samples).

with the URA3 assay,[14] are that it does not require a replica-plating step and that yeast containing mutant *APC* can be visualized as red colonies. These permit analysis of a large number of samples and many clones per sample in a short period. We have confirmed clonal *APC* gene mutations in both mRNA and genomic DNA of the clinical samples that gave red colonies of >15%. This sensitivity is comparable to that of the yeast functional assay for *p53* mutation which we previously reported.[17] It indicates that the present assay is applicable to the clinical tumor samples which may be even contaminated with a variable amount of normal tissues, whereas the URA3 assay could not be applicable, because the assay requires a replica-plating step in the selection of yeast clones and could fail to detect a mutant of a small fraction in the tumor tissue contaminated with normal host cells.[14]

Most of colon cancer cell lines gave 96 to 99% of red colonies in one of five regions. Although HT-29 cells gave approximately 30% and 70% of red colonies in respective regions II and IV, this can be explained from the cytogenetic finding that karyotype of this cell line is hypertriploid (ATCC no.: HTB-38). The percentages of red colonies by colon and breast cancers, which were demonstrated to harbor the *APC* mutations, ranged from 15 to 57% in regions I to V. The relatively low percentage of red colonies in tumor tissues compared to those of cell lines is presumed not because of reduced amounts of mutant *APC* mRNA (nonsense mediated decay),[23] from the almost equivalent percentages of the red colonies given by the tests on the mRNA and the corresponding genomic DNA. It is most conceivable to be because of admixture of cells containing wild-type *APC* alleles, ie, normal cells and tumor cell clone(s) that do not acquire *APC* mutation.

We screened 23 colon cancers by the yeast color assay and found 15 *APC* mutations in 13 cancers (57%). Eight of 16 (50%) somatic mutations were located in the mutation cluster region (MCR, codon 1286 to 1513). This finding is consistent with previous reports that >60% of the somatic mutations were clustered within the MCR.[9] In contrast to the findings in colon cancers, the majority of mutations found in breast cancers were scattered on the entire *APC* coding region and only 4 of 15 (27%) were located in the MCR, indicating that the concept of a mutation cluster region of the *APC* is not applicable to breast cancer. Powell et al[24] presented evidence that *APC* mutations occur early during colorectal tumorigenesis. Our data of colon cancers reconfirmed their findings, by demonstrating the *APC* gene mutation at any of the clinical stages of colon cancer. In breast cancers, however, mutations were significantly more frequent in higher, advanced stages, suggesting that the *APC* mutations are associated with tumor progression in breast cancers. The *APC* inactivation may lead to suppressed degradation of β-catenin.[18] Because Tcf-4 protein was immunohistochemically detected at a high level in restricted tissues such as the intestinal and mammary gland epithelium and carcinomas derived therefrom,[25] a constitutively active Tcf-4/β-catenin complex that is a consequence of the *APC* mutations may result in the uncontrolled transcription of its target genes.[26,27] It is noteworthy that *APC* mutations were detected mainly in solid tubular (7 of 25) or papillary tubular (5 of 25) breast carcinomas but only one mutation was detected in 14 scirrhous-type carcinomas. It might be possible that *APC* mutation plays differential roles in different types of breast cancers. Alternatively, it is possible that the lower rate of *APC* mutations in the scirrhous tumors might be because of the higher percentage of normal cells that may conceal a mutant APC within the background level of the APC yeast color assay.

It has been reported that most identified base substitutions in the *APC* gene are changes from C to T.[28] In the present study, a similar trend was observed in colon cancers, whereas G to T change was dominant in breast cancers. At present it is not clear whether the difference of mutation pattern between the colon and breast cancer reflects an intrinsic (endogenous) pattern of mutation or exposures to a particular environmental mutagen(s). Furthermore, racial and/or environmental factors cannot be excluded. The age-adjusted breast cancer incidence rate and mortality rate of Japanese women are three to four times lower than those of most Western women.[29,30] To elucidate the difference, it is necessary to detect *APC* mutations in breast cancers from ethnically different populations; the *APC* yeast color assay will provide a powerful means for detection of the *APC* mutations in such clinical samples.

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