Jpn. J. Cancer Res. 85, 147–151, February 1994

Lower Incidence of K-*ras* Codon 12 Mutation in Flat Colorectal Adenomas than in Polypoid Adenomas

Seiichi Yamagata,[1] Tetsuichiro Muto,[1] Yoshihiro Uchida,[2] Tadahiko Masaki,[1] Toshio Sawada,[1] Nelson Tsuno[1] and Takashi Hirooka[3]

[1]First Department of Surgery, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, [2]Department of Surgery, Tokyo Metropolitan Toshima Hospital, 33-1 Sakae-cho, Itabashi-ku, Tokyo 173 and [3]Kishiwada-Tokusukai Hospital, 4-22-38 Isogami-cho, Kishiwada-shi, Osaka 596

In order to clarify genetic changes in flat adenomas, K-*ras* codon 12 point mutations were examined in 56 flat adenomas, 81 polypoid adenomas and 42 cancers of colon and rectum. The mutation frequency in flat adenomas was 23% (13/56), significantly lower than that in polypoid adenomas (67%: 54/81) and cancers (76%: 32/42). Even mildly dysplastic adenomas or small (less than 5 mm) adenomas showed higher mutation incidence in polypoid type (62%, 57%) than in flat type (23%, 19%). Among flat adenomas, flat elevated lesions exhibited relatively higher mutation frequency than completely flat or depressed ones. As for cancers, 14 tumors (33%) contained mutations only in a minor tumor cell population, indicating that these mutations occur at a late stage of tumorigenesis. These results suggest that the adenoma-carcinoma sequence through flat adenomas may be different from that through polypoid adenomas, and genetic changes may be heterogeneous in colorectal carcinogenesis.

Key words: K-*ras* — Flat adenoma — Colorectal neoplasm — Adenoma-carcinoma sequence

Several oncogenes and tumor suppressor genes are associated with human colorectal carcinogenesis.[1-9] Vogelstein *et al.*[5] reported the accumulation of genetic alterations during the adenoma-carcinoma sequence of colorectal carcinogenesis and suggested that *ras*-gene mutations are relatively early events in colorectal tumorigenesis. Conversely, a study of familial adenomatous polyposis revealed that K-*ras* codon 12 mutation frequency in adenomas with severe atypia was higher than in advanced cancers.[10] Furthermore, Fujimori *et al.*[11] showed that no K-*ras* codon 12 point mutation could be detected in the depressed type of superficial early colorectal cancers. These studies suggested that another pathway to advanced colorectal carcinoma, not involving K-*ras* codon 12 mutation, might exist.

The past several years have witnessed increasing incidence of flat adenoma as a new precursor of colorectal carcinoma.[12-17] Flat adenoma is unique in morphology, with dysplastic tubules spreading superficially at its margin and occupying the entire mucosal layer at its center. The height of the lesion is not greater than twice the thickness of the adjacent normal mucosa. Histologically, flat adenomas are more likely to exhibit a high grade of epithelial dysplasia than polypoid adenomas and they are considered to play an important role in colorectal tumorigenesis.[12, 13, 16, 17] In this study, we analyzed K-*ras* codon 12 mutations in flat adenomas polypoid adenomas and advanced cancers, using a sensitive PCR method.[18] The role of flat adenomas in colorectal carcinogenesis is discussed.

MATERIALS AND METHODS

Materials Fifty-six flat adenomas, 81 polypoid adenomas, and 42 cancers were resected surgically or endoscopically from 171 patients in our institutions. All specimens were fixed in formalin and embedded in paraffin. From these blocks, 5-μm sections were cut for DNA extraction and adjacent 3-μm sections were stained with hematoxylin and eosin for histological examination.

Histological examination Pathological analyses were performed independently by one of the authors (T.M.) without knowing the status of K-*ras* codon 12 mutation. The atypia of adenomas was graded as mild, moderate, or severe according to the WHO's criteria.[19] Intramucosal cancers were included in adenomas with severe atypia.

Flat adenomas were defined as previously described.[17] Furthermore, they were classified by their microscopic appearances into the following subgroups — IIa type: flat elevation (n=22) (Fig. 1A), IIb type: completely flat lesion (n=15) (Fig. 1B), IIc type: slightly depressed lesion (n=7) (Fig. 1C), and combined types: IIa+IIc type (n=5), IIb+IIa type (n=2). Five flat adenomas could not be classified because they were not cut adequately. The relationship between these morphological types and K-*ras* mutations were examined.

DNA preparation An area of approximately 10 mm^2, precisely corresponding to the dysplastic lesion, was cut out from the section under a microscope and digested with Proteinase K after deparaffinization.[20] Each area

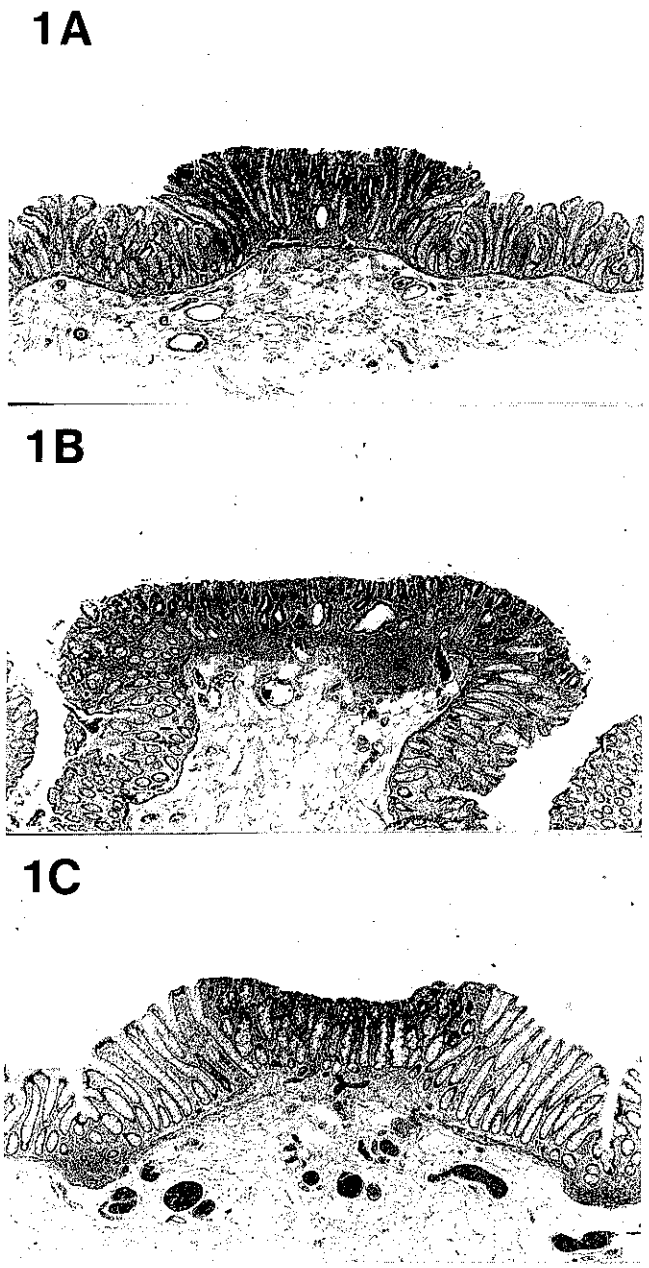

Fig. 1. Subgroups of flat adenomas. A: a flat elevated lesion (IIa type); B: a completely flat lesion (IIb type); C: a slightly depressed lesion (IIc type).

comprised 10^4–10^5 cells and more than 80% of them were dysplastic cells. The DNA was purified by phenol-chloroform extraction and ethanol precipitation, and one-tenth of it was subjected to polymerase chain reaction (PCR).

Two-step sensitive PCR The DNA was amplified as previously described[18] with minor modifications. The PCR mixture was 50 μl for each sample, containing the genomic DNA, 1.25 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT), deoxyribonucleotide triphosphates at 200 μM each, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 12.5 pmol of each primer. The samples were subjected to 40 cycles of amplification, using an automatic thermal controller (MJ Research Inc., Watertown, MA). Each cycle consisted of 2 min at 95°C, 3 min at 55°C, and 1.5 min at 72°C. The primers used for PCR were as follows:

A(forward) 5′ACTGAATATAAACTTGTGGT-AGTTGGACCT3′
B(reverse) 5′GTCCTGCACCAGTAATATGC3′
C(reverse) 5′CTATTGTTGGATCATATTCG3′

The underlined base of primer A represents a mismatch from the K-*ras* DNA sequence which creates an *Mva*I recognition site if codon 12 of template DNA is normal.

The first PCR was performed with primers A and B, generating 147-base pair fragments, containing an *Mva*I recognition site if codon 12 is normal. Seventeen μl of the product was digested with 10 units of *Mva*I (Takara Shuzo, Kyoto) and 0.5 μl of it was re-amplified by a second PCR with primers A and C, generating 106-base pair fragments, also containing an *Mva*I site if codon 12 is normal. Then, 8 μl of the second PCR product was digested with 10 units of *Mva*I and electrophoresed on a 8% acrylamide gel. Negative controls (no DNA and normal DNA) were run with each analysis.

Titration experiment A549 is a lung cancer cell line that contains a glycine-to-serine mutation at codon 12 of c-K-*ras* and lacks the normal allele.[21] Genomic DNAs from A549 cell line and normal gallbladder were mixed in various ratios and analyzed to estimate the sensitivity of the 2-step sensitive PCR method.

Furthermore, we determined the point of equivalence (the dilution ratio at which the intensities of the wild-type band and mutant band were equal), and quantitated approximately the ratio of mutant in each sample.

RESULTS

The frequency of c-K-*ras* mutations at codon 12 in colorectal flat adenomas, polypoid adenomas, and advanced cancers is shown in Fig. 2. Only 23% (13/56) of flat adenomas contained K-*ras* mutations, whereas 67% (54/81) of polypoid adenomas contained the mutations. There was a significant difference of K-*ras* mutation frequency between flat adenomas and polypoid adenomas ($P<0.01$, chi-square test). Cancers contained K-*ras* mutations slightly more frequently than polypoid adenomas (76% of 42 tumors). The frequency of K-*ras* mutations in flat adenomas with mild or moderate atypia was sig-

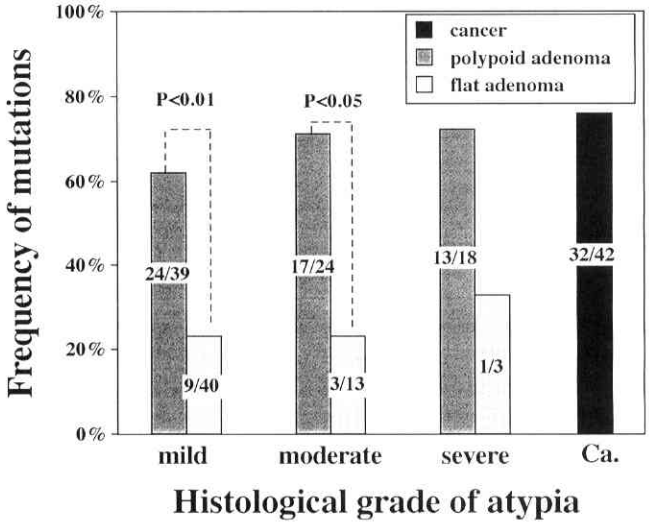

Fig. 2. Frequencies of K-*ras* codon 12 mutation relative to histological grade of atypia in colorectal tumors.

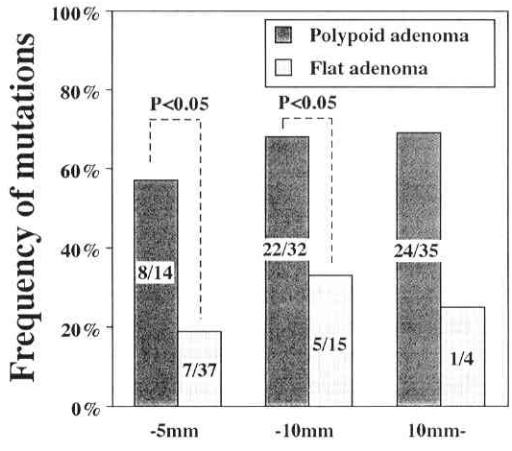

Fig. 3. Correlation between size of adenomas and K-*ras* codon 12 mutations.

nificantly lower than that in polypoid adenomas with the same grade of atypia (mild: 23% (9/40) vs. 62% (24/39) $P<0.01$, moderate: 23% (3/13) vs. 71% (17/24) $P<0.05$).

It is noteworthy that 57% (8/14) of small (less than 5 mm) polypoid adenomas contained mutations, while 19% (7/37) of small flat adenomas did so ($P<0.05$) (Fig. 3). As for medium-sized adenomas (6–10 mm), flat and polypoid type also showed different frequency of ras mutations (33% and 68%, respectively).

Table I. Morphological Type and K-*ras* Mutation in Flat Adenoma

Morphological type	Frequency of mutation	
IIa	7/22	9/24 (38%)
IIb+IIa	2/2	
IIb	2/15	2/22 (9%)
IIc	0/7	
IIc+IIa	0/5	
Unclassified	2/5	

$P<0.05$

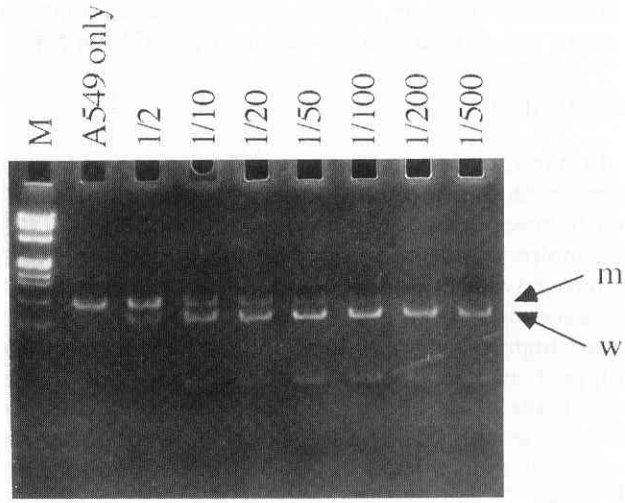

Fig. 4. Titration experiment using the 2-step sensitive PCR method. Genomic DNA from the A549 cell line was diluted with that from normal human gallbladder in the ratios indicated. Arrows (right) indicate the positions of mutant (m) and wild-type (w) bands. The mutant band was relatively weak when diluted at more than 1:20 ratio but was detected until 1:100 dilution. Lane M: Øx174/*Hae*III DNA marker.

Elevated types of flat adenomas (IIa type and IIb+IIa type) showed relatively high incidence of K-*ras* codon 12 mutations (9/24: 38% in total). Only 2 of the IIb type adenomas contained K-*ras* codon 12 mutations and none of the IIc type adenomas showed K-*ras* mutation. The frequency of K-*ras* mutations in these non-elevated flat adenomas was 9% (2/22), which was significantly lower than that in flat elevated adenomas ($P<0.05$). No mutation was detected among 5 adenomas of IIc+IIa type (depressed adenoma with surrounding elevation) (Table I).

By a titration experiment, the sensitivity of the 2-step PCR method was revealed to be about 1/100 (Fig. 4). The intensity of the mutant band was nearly equal to that of the wild type band at 10- to 20-fold dilution (the point of equivalence) and was obviously weaker when the ratio

Table II. K-*ras* Codon 12 Mutation in Colorectal Tumors Relative to the Ratio of Mutant

Histology	Total	Positive for the mutation		
		The ratio of mutant		Total
		<5%	≥5%	
Flat adenoma	56	7	6 (11%)	13 (23%)
Polypoid adenoma	81	15	39 (48%)	54 (67%)
Cancer	42	14	18 (43%)	32 (76%)

of the mutant was less than 1/20. Such weak 106-bp bands were observed in 7 flat adenomas, 15 polypoid adenomas and 14 cancers, and the ratio of the mutant in these tumors was estimated to be less than 5% (Table II).

DISCUSSION

In our study, the frequency of K-*ras* codon 12 mutations in 56 flat adenomas was 23%, which was significantly lower than that in polypoid adenomas ($P<0.01$). It is interesting to note that grade of atypia does not seem to have any effect on K-*ras* mutation rate in polypoid or flat adenomas. Even small polypoid adenomas showed a rather high frequency of K-*ras* mutations, indicating that polypoid adenomas may acquire mutations at a very early stage of development. Among flat adenomas, flat elevated lesions (IIa type) showed relatively higher frequency than completely flat or depressed lesions (IIb or IIc type). It can be assumed that IIa type occupies an intermediate position between polypoid adenomas and IIb or IIc type, with regard to K-*ras* mutations as well as morphological classification. Considering that superficial early colorectal cancers were reported to manifest no K-*ras* mutation,[11] it can be assumed that IIb type or IIc type adenomas may be precursors of superficial early cancers. On the other hand, Lynch *et al.*[14] speculated that flat adenomas may be merely an early stage of small polypoid adenomas. The IIa type may include such kinds of adenomas, but IIb type and IIc type are certainly different from polypoid adenomas. From these observations, it can be said that the presence or absence of K-*ras* codon 12 mutation correlates with the shape of colorectal adenomas.

The mutation frequency in colorectal cancers was very high (76%) but this does not imply that most of them developed from polypoid adenomas with K-*ras* codon 12 mutations. If they developed from such adenomas, they should consist mostly of tumor cells containing K-*ras* mutations. However, the mutation was detected only in a minority of cells in 33% of the cancers, indicating that the mutations occurred in a later stage of carcinogenesis. This heterogeneity in colorectal cancers was also reported by Finkelstein *et al.*[22] They showed that approximately 10% of primary colorectal cancers expressed the point mutation exclusively in deeper, invasive portions. K-*ras* codon 12 mutations were infrequent in flat adenomas, but it cannot be denied that flat adenomas grow to cancers, acquiring K-*ras* mutations in a minority of their constituent cell population. Further investigations on flat or depressed cancers that are considered to have originated from flat or depressed adenomas seem essential.

Several methods to detect K-*ras* mutations have been reported so far. The PCR-ASO (allele specific oligonucleotide hybridization) assay and PCR-direct sequence method are often used, but these methods can not detect the mutations until at least 10%–20% of cells contain them.[22, 23] On the other hand, the 2-step sensitive PCR method can selectively amplify the mutant so that the sensitivity is very high (1/100 in our study and 1/500 according to Levi *et al.*[18]). Considering the different sensitivity, it may be reasonable that the mutation frequency in polypoid adenomas and cancers was found to be relatively higher in our study than in previous studies. As shown in Table II, considerable numbers of tumors contained the mutations only in a very small population of cells. If such genetic changes in a minority of cells were not detected, the mutation frequency would be 11% in flat adenomas, 48% in polypoid adenomas, and 43% in advanced cancers. Nevertheless, it is important that K-*ras* codon 12 mutation in flat adenomas, especially IIb type and IIc type, was infrequent even when examined by the 2-step sensitive PCR method.

This study focused exclusively on K-*ras* codon 12 mutations. However, a number of specific gene alterations are assumed to be associated with colorectal tumorigenesis. An integrated genetic approach to superficial type of adenoma should lead to clarification of a new pathway of colorectal tumorigenesis.

ACKNOWLEDGMENTS

We thank Dr. Shoji Kuwata for critical advice and Mrs. Masako Kuwabara for her excellent technical assistance. A549 cell line was provided by the Japanese Cancer Research Resources Bank (Tokyo). This study was financially supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan.

(Received September 8, 1993/Accepted November 9, 1993)

REFERENCES

1) Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J. and Vogelstein, B. Prevalence of *ras* gene mutations in human colorectal cancers. *Nature,* **327,** 293–297 (1987).
2) Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and Perucho, M. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature,* **327,** 298–303 (1987).
3) Burmer, G. C. and Loeb, L. Mutations in the KRAS2 oncogene during progressive stage of human colon carcinoma. *Proc. Natl. Acad. Sci. USA,* **86,** 2403–2407 (1989).
4) Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E., Simons, J. W., Ruppert, J. M., Hamilton, S. R., Preisinger, A. C., Thomas, G., Kinzler, K. W. and Vogelstein, B. Identification of a chromosome 18q gene that is altered in human colorectal cancers. *Science,* **247,** 49–56 (1990).
5) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Eng. J. Med.,* **319,** 525–532 (1988).
6) Kinzler, K. W., Nilbert, M. C., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hamilton, S. R., Hedge, P., Markham, A., Carlson, M., Joslyn, G., Groden, J., White, R., Miki, Y., Nishisho, I. and Nakamura, Y. Identification of a gene located at chromosome 5q21 that is mutated in colon cancers. *Science,* **251,** 1366–1370 (1991).
7) Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKecnie, D., Finniear, R., Markham, A., Groffen, J., Bogusuki, M. S., Altshul, S. F., Horii, A., Ando, H., Miyoshi, W., Miki, Y., Nishisho, I. and Nakamura, Y. Identification of FAP locus genes from chromosome 5q21. *Science,* **253,** 661–665 (1991).
8) Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. p53 mutations in human cancers. *Science,* **253,** 49–53 (1991).
9) Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Bada, S., Hedge, P., Markham, A., Krush, A. J., Peterson, G., Hamilton, S. R., Nilbert, M. C., Levy, D. B., Bryan, T. M., Preisinger, A. C., Smith, K. J., Su, L.-K., Kinzler, K. W. and Vogelstein, B. Mutations in 5q21 genes in FAP and colorectal cancer patients. *Science,* **253,** 665–669 (1991).
10) Ando, M., Maruyama, M., Oto, M., Takemura, K., Endo, M. and Yuasa, Y. Higher frequency of point mutations in the c-K-*ras* 2 gene in human colorectal adenomas with severe atypia than in carcinomas. *Jpn. J. Cnacer Res.,* **82,** 245–249 (1991).
11) Fujimori, T., Nagasako, K., Satonaka, K., Hirayama, D., Harada, M., Muratani, A., Tabata, T., Kitazawa, S., Ikehara, H. and Maeda, S. Expression of *ras* oncogene product (p21) and point mutation of Ki-*ras* codon 12 in superficial early colorectal cancer. *I to Cho,* **27,** 111–117 (1992) (in Japanese).
12) Adachi, M., Muto, T., Morioka, Y., Ikenaga, T. and Hara, M. Flat adenoma and flat mucosal carcinoma (IIb Type) — a new precursor of colorectal carcinoma? *Dis. Colon Rectum,* **31,** 236–243 (1988).
13) Adachi, M., Muto, T., Okinaga, K. and Morioka, Y. Clinicopathologic features of flat adenoma. *Dis. Colon Rectum,* **34,** 981–986 (1991).
14) Lynch, H. T., Smyrck, T. C., Watson, P., Lanspa, S., Lynch, P. M., Jenkins, J., Rouse, J., Cavalieri, J., Howard, L. and Lynch, J. Hereditary flat adenoma syndrome: a variant of familial adenomatous polyposis? *Dis. Colon Rectum,* **35,** 411–421 (1992).
15) Lanspa, S. J., Rouse, J., Smyrk, T., Watson, P., Jenkins, J. X. and Lynch, H. T. Epidemiologic characteristics of the flat adenoma of Muto. *Dis. Colon Rectum,* **35,** 543–546 (1992).
16) Muto, T., Kamiya, J., Sawada, T., Konishi, F., Sugihara, K., Kubota, Y., Adachi, M., Agawa, S., Saito, Y., Morioka, Y. and Tanpryoon, T. Small "flat adenoma" of the large bowel with special reference to its clinicopathologic features. *Dis. Colon Rectum,* **28,** 847–851 (1985).
17) Wolber, R. A. and Owen, D. A. Flat adenoma of the colon. *Hum. Pathol.,* **22,** 70–74 (1991).
18) Levi, S., Urbano-Ispizua, A., Gill, R., Thomas, D. M., Gilbertson, J., Foster, C. and Marshall, C. J. Multiple K-*ras* codon 12 mutations in chorangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res.,* **51,** 3497–3502 (1991).
19) Morson, B. C. and Sobin, L. H. "Histological Typing of Intestinal Tumors" (1976). World Health Organization, Geneva.
20) Goelz, S. E., Hamilton, S. R. and Vogelstein, B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Bichem. Biophys. Res. Commun.,* **130,** 118–126 (1985).
21) Valenzuela, D. M. and Groffen, J. Four human carcinoma cell lines with novel mutations in position 12 of K-*ras* oncogene. *Nucleic Acids Res.,* **14,** 843–852 (1986).
22) Finkelstein, S. D., Sayegh, R., Christensen, S. and Swalsky, P. A. Genotypic classification of colorectal adenocarcinoma. *Cancer,* **71,** 3827–3838 (1993).
23) Smit, V. T. H. B. M., Boot, A. J. M., Smits, A. M. M., Fleuren, G. J., Cornelisse, C. J. and Bos, J. L. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinoma. *Nucleic Acids Res.,* **16,** 7773–7782 (1988).