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Frequent Altered Expression of Fragile Histidine Triad Protein in Human Colorectal Adenomas

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Fragile histidine triad (*FHIT*) gene is involved in deletions on the short arm of chromosome 3 in various human cancers. We found that 47% of colorectal adenomas, which is a higher frequency than that of K-*ras*, showed altered expression of the Fhit protein by Western blot analysis. The amount of Fhit protein was inversely correlated with the degree of dysplasia. Importantly, 27% of low-grade dysplastic adenomas showed altered expression of Fhit protein. Additionally, expression of human Fhit protein in human colon carcinoma cell line SW480 exhibited a marked inhibition of growth and rendered SW480 cells highly susceptible to undergo apoptosis compared with control cells. These findings suggest that altered expression of the *FHIT* gene is a quite early aberration in the development of colorectal tumors and that Fhit protein may act as a tumor suppressor.

Key Words: **Fhit; tumor suppressor; colorectal adenoma; growth inhibition; apoptosis.**

Recently the *FHIT* gene was mapped by positional cloning of t(3;18) chromosomal translocation in renal cancer to chromosome 3p14.2, a location encompassing *FRA3B*, which is the most active constitutive chromosomal fragile site known (1). *FHIT* encodes a 147-amino acid protein that has a HIT (histidine triad) sequence motif identified by the presence of a histidine triad, HXHXHX, where is a hydrophobic residue (1). Fhit protein has diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) hydrolase activity that cleaves Ap_3A into adenosine 5′-diphosphate and adenosine 5′-phosphate *in vitro* (2). Aberrant transcripts and loss of Fhit protein have been observed in various kinds of tumor cells, such as human lung, stomach, head, and neck (3–5). Abnormal expression of the Fhit protein in lung is associated with smoking history and its prognosis. Interestingly, alterations in the *FHIT* gene were found to occur at much higher rates (80% vs 22%) in tumors from heavy smokers (6). This finding suggests that *FHIT* may be a ready target for carcinogens present in cigarette smoke and thereby be associated with the initiating process of malignant transformation as a tumor suppressor. The mutant Fhit protein, which does not have Ap_3A hydrolase activity, functioned as tumor suppressor, indicating that Ap_3A hydrolase activity is not necessary for its tumor suppressor activity (7). Colon cancer is also thought to be initiated by exposure to dietary carcinogens that chronically affect the glandular cells of the colorectal mucosa. Hao *et al.* reported that 44% of 84 colorectal carcinomas showed a marked loss or absence of Fhit expression (8). Here we examined Fhit protein expression in colorectal adenomas, which are premalignant lesion of the colon, and found that the loss or reduction of Fhit expression occurred in a large fraction of colorectal adenomas by Western blot analysis. Furthermore, we were able to establish stable cell lines expressing human Fhit protein and investigated its tumor suppression activity.

MATERIALS AND METHODS

Tissue preparation. All human tissue samples (colorectal adenomas and their adjacent normal mucosa from specimens resected for adenoma) were specimens obtained with informed consent from patients who were undergoing biopsies at Osaka Medical College, Takatsuki, Osaka, between 1999 and 2000. All tissues were divided into cancerous and normal parts. We examined 30 cases of colorectal adenomas. For each tissue sample, a small part was applied for Western blot analysis and DNA extraction, and the remainder was fixed in 10% buffered formaldehyde solution and embedded in paraffin. One section was stained with hematoxylin-eosin and reviewed by two pathologists to confirm the diagnosis including pathological status. The adenomas were classified according to the International Histological Classification of Tumours. Low-grade dysplasia includes mild and moderate dysplasia, and high-grade dysplasia includes

Abbreviation used: RT-PCR, reverse transcription-polymerase chain reaction.

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severe dysplasia. For immunohistochemical study, four series of 4-μm-thick sections were prepared. The other sections were used for analyzing the expression of Fhit proteins.

Western blot analysis. Fresh tissues were homogenized in lysis buffer (2 × PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxychorlate, and 0.1 mM phenylmethanesulfonyl fluoride). The homogenized samples were centrifuged, and the clear supernatant was used as protein lysate. The protein concentration was determined by the method of Markwell *et al.* (9). Two micrograms of lysate protein was separated by SDS-PAGE using a 12% polyacrylamide gel and electroblotted onto a PVDF membrane (DuPont, Boston, MA). After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4°C with anti-human Fhit antibody (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:300. The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-rabbit antibody (New England Biolabs, Beverly, MA) at room temperature, and then washed three times with TPBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs).

Immunohistochemical staining. The 4-μm sections from paraffin-enbedded tissues were mounted on poly-L-lysine-coated slides. They were then deparaffinized in xylene and dehydrated with graded ethanol. Endogenous peroxidase activity was blocked with 0.2% (w/v) sodium azide containing 0.3% (v/v) hydrogen peroxide in graded methanol for 60 min. After having been washed in 0.01 M phosphate-buffer saline (PBS), the section was blocked for non-specific binding with Avidin/Biotin Blocking solution (Vector Laboratories, Inc., CA). The sections were then incubated with rabbit polyclonal anti-Fhit antibody diluted at 1:1000 in a moist chamber overnight at 4°C. After having been washing in 0.01 M PBS, the sections were subsequently incubated for 30 min at room temperature with biotinylated goat anti-rabbit immunoglobulin for Fhit expression. After another wash in PBS, peroxidase-conjugated streptavidin (Vector Stain Elite ABC Kit, Vector Laboratories) was applied, and the sections were again incubated for 30 min. After washing out of the excess complex, the localization of immunoreactive complexes was visualized by incubation of the sections for 5–10 min in 0.05 M Tris-HCl (pH 7.6) containing 0.02% (w/v) 3-3′-diaminobenzidine tetrahydrochloride and 0.03% (v/v) hydrogen peroxide. A negative control section, to which normal rabbit preimmune serum was applied in slides for Fhit expression, was included in each staining. Counter staining was performed with hematoxylin.

Single-strand conformation polymorphism (SSCP) analysis of K-ras codon 12. DNA was extracted from colorectal adenoma tissue in each patient. The oligonucleotides that were used as primers for K-*ras* codon 12 were as follows: sense; 5′-GACTGAATATAAACTTGTGG-3′ and antisense; 5′-CTATTGTTGGATCATATTCG-3′ (Takara Shuzo Company, Ltd., Kyoto, Japan). The PCR reaction consisted of 30 cycles (94°C for 30 s, 57°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). 2-μl aliquots of PCR products were mixed with formamide, heated and subjected to electrophoresis for 1.5 h using 12% polyacrylamide minigels. The gels were then silver stained and single-strand DNA fragments were visualized directly.

Construction of expression vector for human Fhit. A human *FHIT* cDNA clone was cloned by RT-PCR method using RNA from peripheral lymphocytes; and the resultant 455-bp *Bam*HI fragment including the entire coding region was inserted into a pIRES1*neo* vector of 5.3 kb. The pIRES1*neo* vector, derived from pCIN4, contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus, and permits the translation of two open reading frames, *FHIT* and *neomycin phosphotransferase II* (*NPT II*) cDNA, from one messenger RNA (10). Cells expressing a high level of Fhit protein can be selected by neomycin resistance. The final construct, designated pIRES-*FHIT*, was used for gene transfection. The pIRES1*neo* vector, was used for transfection as control.

Transfection of colon carcinoma cell line SW480 cells and establishment of human Fhit expressants. Cells of the human colon carcinoma cell line SW480 which has K-*ras* and *p53* mutations (11) were cultured in RPMI 1640 medium supplemented with 10% FBS. Exponentially growing cells were transfected with the plasmid by the method using LipofectAMINE (GibcoBRL, Rockville, MD). Briefly, the cells (5×10^5 cells/60-mm dish) were cultured overnight and then incubated with liposome-entrapped pIRES-*FHIT* or pIRES1*neo* (1 μg DNA/100 nmol of lipids in 1 ml of the medium). After incubation for 16 h, the cells were cultured in the fresh medium for one day and then selected with neomycin (G418) at the concentration of 1000 μg/ml with exchanging for the same medium every three days. After selection for seven days, the living cells were segregated by limiting dilution. Two clones, which were shown by RT-PCR to express human *FHIT* mRNA, were obtained at 21 days after transfection. Briefly, total cellular RNA was isolated by the phenol/guanidium thiocyanate method with DNase I treatment (10). After reverse transcription of 2 μg of total RNA, cDNA was generated. PCR primers that were used to amplify the cDNA sequences were as follows: for *FHIT*, sense; 5′-CATCCTGGAAGCTTTGAAGC-3′ and antisense; 5′-CTGGTTGAAGAATACAGGATGG-3′. The human glyceraldehyde phosphate dehydrogenase (*GAPDH*) product was used for an internal standard. The PCR products were analyzed by electrophoresis on 2% agarose gels. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). The PCR products were analyzed by electrophoresis on 2% agarose gels. Furthermore, the Fhit protein expression was examined by Western blot analysis.

Evaluation of cell growth characteristics. Cells in mass culture or in colonies developing from sparsely seeded cells were inspected for cell number and morphological changes by phase-contrast microscopy. Cell viability was assessed by a dye exclusion test.

Assessment of apoptosis by exposure to hydrogen peroxide. Hydrogen peroxide diluted with PBS was used at various concentrations. For morphological examination of apoptosis, cells were stained with Hoechst 33342 (5 μg/ml) at 37°C for 30 min, washed twice with PBS, pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) equipped with an epi-illuminator and appropriate filters. For analysis of DNA fragmentation, cellular DNA was extracted from whole cells. RNase was added to the DNA solution at the final concentration of 20 μg/ml, and the mixture was incubated at 37°C for 30 min. After electrophoresis on a 2.5% agarose gel, DNA was visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

The histopathological and clinical findings on 30 tissue samples of colorectal adenomas are summarized in the Table 1. The median age of the 30 patients was 66 years (range: 39–77 years), and the subjects included 22 men and 8 women. None had received radiation or chemotherapy prior to resection. We classified adenomas into 2 types, L (low-grade dysplasia) and H (high-grade dysplasia) by pathological findings, as shown in the Table 1. The expression of Fhit in 30 colorectal adenomas was firstly examined by Western blot analysis using anti-human Fhit antibody. From the results obtained by Western blot analysis, we classified the adenomas into 3 types, i.e., preserved (P), reduced (R), and absent (A) according to the amount of Fhit protein detected (Fig. 1). As shown in the Table 1, Fhit expression was retained in 16 of 30 (P, 53%) adenomas, was

TABLE 1

Clinicopathologic Features and Expression of Fhit Protein in Human Colorectal Adenomas

Case	Age	Sex[1]	Size[2]	Site[3]	Morphology[4]	Histology[5]	Fhit[6]	K-ras[7]
1	50	F	15	D	Ip	H	R	(−)
2	69	M	10	D	Ip	H	P	(−)
3	39	M	13	R	Isp	H	R	(−)
4	59	M	9	D	Ip	H	R	(+)
5	72	M	4	S	Is	L	R	(−)
6	71	M	6	A	Isp	H	A	(−)
7	56	F	9	C	IIa	L	P	(+)
8	77	M	10	S	IIa	H	A	(−)
9	66	M	13	T	IIa	H	A	(+)
10	73	M	6	S	Ip	L	R	(−)
11	57	F	5	A	Isp	L	R	(−)
12	60	M	15	T	IIa	H	P	(−)
13	66	M	10	D	Ip	H	R	(−)
14	59	M	10	S	Ip	H	A	(+)
15	57	F	4	S	Isp	L	P	(−)
16	63	M	8	A	Isp	H	R	(+)
17	66	M	5	D	Isp	L	P	(−)
18	58	F	7	R	Isp	L	P	(−)
19	69	F	5	S	Ip	L	P	(−)
20	66	M	7	T	Isp	H	P	(−)
21	61	M	6	D	Ip	L	P	(−)
22	69	M	9	A	Isp	L	P	(+)
23	76	M	4	A	Isp	L	P	(−)
24	63	F	7	S	Ip	H	P	(−)
25	73	M	13	R	Isp	H	R	(+)
26	70	M	10	R	IIa	H	P	(−)
27	52	M	6	D	Is	L	P	(−)
28	46	M	5	S	Isp	L	P	(−)
29	70	M	5	T	Is	L	P	(−)
30	76	F	7	D	Ip	L	R	(−)

Summary of Fhit Protein Expression

	Fhit protein expression		
	Preserved	Reduced or absent	Total
Adenomas			
High[a]	5	10	15
Low[b]	11	4	15
Total	16	14	30

Note. FHIT expression is associated with dysplasia: $P < 0.05$.

[a] High, adenoma with high-grade dysplasia.

[b] Low, adenoma with low-grade dysplasia.

[1] M, male; F, female. [2] Diameter in mm. [3] Location of tumor; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum. [4] Ip, pedanculate; Isp, subpedunculate; Is, sessile; IIa, superficial elevated. [5] H, adenoma with high-grade dysplasia; L, adenoma with low-grade dysplasia. [6] P, preserved; R, reduced; A, absent. [7] (+), mutation positive; (−), mutation negative.

reduced in 10 of 30 (R, 33%), and was almost absent in 4 of 30 (A, 13%). We also examined its expression by immunohistochemistry in some cases of each type, with the result that the amount of immuno-reactive complex of Fhit distributed heterogeneously in adenoma cells of the reduced cases was less, and never found in the absent cases compared with the level for the preserved cases (data not shown). The amount of Fhit immunocomplex was in good agreement with the results obtained by the Western blot analysis.

There was a negative correlation between the amount of Fhit protein and the degree of dysplasia in adenomas (χ^2 test, $P = 0.028$) (Table 1). Importantly, altered expression of Fhit protein was found in 4 of the 15 low-grade dysplasia cases (27%). The K-*ras* codon 12 mutation detected by PCR with single-strand conformation polymorphism analysis was found in 7 of the 30 (23%) adenomas (Table 1). Five of the 7 cases showing the K-*ras* mutation were the high-grade dysplasias. All high-grade dysplasias cases which had the

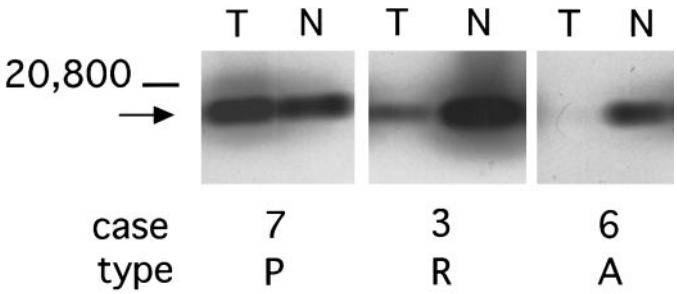

FIG. 1. Western blot analysis of Fhit protein in colorectal adenoma and its adjacent normal mucosa. N (control) indicates normal mucosal tissues of patients 7, 3, and 6 respectively; and T in matched samples from three patients indicates tumor tissues of patients 7 (P), 3 (R), and 6 (A) respectively. An arrow indicates the bands of Fhit protein detected. (P), (R), or (A) indicates preserved, reduced, or almost absent expression of Fhit protein respectively.

K-*ras* mutation showed altered expression of Fhit protein.

We next cloned the *FHIT* cDNA from RNA of human peripheral lymphocytes by RT-PCR method and ligated it into pIRES1*neo* eukaryotic expression vector to obtain a high efficiency of Fhit expressants after selection in neomycin. This construct was used for transfection of a human colon carcinoma cell line SW480 by means of cationic liposomes to investigate the tumor suppression function of Fhit. pIRES1*neo* vector was also employed to transfect SW480 cells for control (SW/IRES). The expression of *FHIT* mRNA and protein was confirmed by RT-PCR (data not shown) and Western blot analysis. The expression of Fhit protein was significantly reduced in SW480 cells (Fig. 2A). We obtained a few positive clones by limiting dilution and selected two clones, which expressed a significant amount of Fhit protein (Fig. 2A). We designated them SW/FHIT-1 and 2, respectively. The cell growth of SW/FHIT-1 and 2 was significantly suppressed in comparison with parent or two SW/IRES cell lines (Fig. 2B). The growth of SW/IRES cells was similar to that of parent cells. Morphologically, the SW/FHIT-1 or 2 cells tended to be oval-shaped, not spindle and seemed unable to make contact with each other regularly like parent or SW/IRES cells (data not shown). Next, we exposed control cell lines and *FHIT* transfectants to hydrogen peroxide at various concentrations for 18 h to examine the sensitivity toward the oxidative stress. The SW/FHIT-1 cells underwent apoptosis, which was identified by Hoechst 33342 nuclear staining (data not shown) and DNA ladder formation, at the concentration of 20 μM, whereas control cells having only vector plasmid did not (Fig. 3). The content of intracellular reduced glutathione and the mRNA level of *NADPH* examined by RT-PCR were almost the same in those cell lines. We also had the same results from human Fhit expressants in guinea pig fibrosarcoma cell line 104C1 (12).

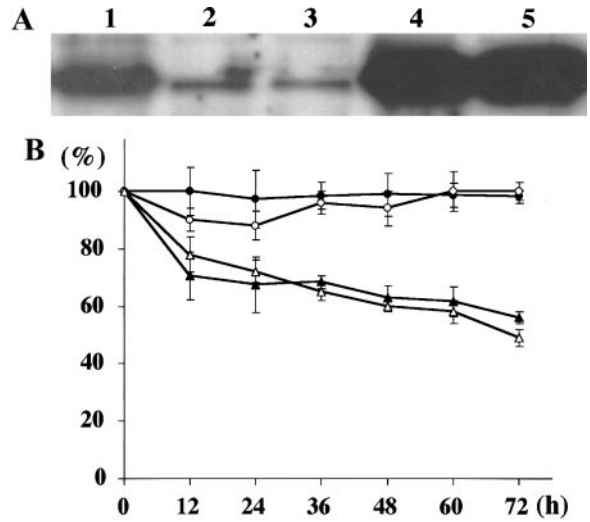

FIG. 2. Effect of additional human Fhit expression on cell growth in a SW480 human colon cancer cell line. (A) Establishment of human Fhit expressants in SW480 cells as judged by Western blot analysis. Lane 1, control human peripheral lymphocytes; lane 2, parent SW480 cells; lane 3, SW/IRES-1 cells; lane 4, SW/FHIT-1 cells; lane 5, SW/FHIT-2 cells. (B) Cell numbers of SW480 cells or transfectants were evaluated by the trypan-blue dye exclusion test and expressed as a percentage of the number in SW480 parent cells. The values were obtained from three independent experiments. ○, SW/IRES-1 cells; ●, SW/IRES-2 cells; △, SW/FHIT-1 cells; ▲, SW/FHIT-2 cells.

Thus, we demonstrated that 47% of colorectal adenomas had a markedly reduced expression of Fhit protein. The frequency of altered Fhit expression was

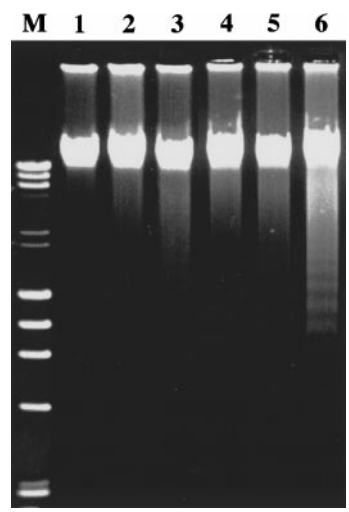

FIG. 3. Nucleosomal DNA fragmentation of SW/IRES-1 or SW/FHIT-1 cells 18 h after exposure to 10 or 20 μM hydrogen peroxide. Three micrograms of DNA was loaded into each lane. Lane 1, SW/IRES-1 cells without treatment; lane 2, SW/IRES-1 cells treated with 10 μM hydrogen peroxide; lane 3, SW/IRES cells treated with 20 μM hydrogen peroxide; lane 4, SW/FHIT-1 cells without treatment; lane 5, SW/FHIT-1 cells treated with 10 μM hydrogen peroxide; lane 6, SW/FHIT-1 cells treated with 20 μM hydrogen peroxide. Lane M is a DNA size marker.

higher than that of K-*ras* mutation and a little lower than that of *APC* in familial or sporadic colorectal adenomas (13, 14). Importantly, the frequency of altered Fhit expression, even in low-grade dysplasias, was approximately 27%. On the other hand, *APC* mutation was observed mainly in the cases with polyps of more than 5-mm in diameter and the majority of them were possibly cases of high-grade dysplasia (13, 15).

The frequent loss or reduction of Fhit protein expression, aberrant *FHIT* transcripts, and numerous deletions within the *FHIT* gene in various tumors suggest that the *FHIT* gene is a tumor suppressor gene common to many cancers (16). Our results provide additional evidence suggesting that the Fhit protein is associated with tumorigenesis in the early stage of the polypoid adenoma-carcinoma sequence of colon cancer. We did not find altered expression of Fhit in any of the matched normal tissues tested. It was reported that mutation of the *APC* gene is found in approximately 60% of sporadic colorectal adenomas and occurs before the K-*ras* mutation (13, 15). Considering that Fhit protein may be a frequent target for carcinogens present in cigarette smoke and that colon cancer may be induced by dietary carcinogens, genomic and/or post-transcriptional abnormalities of *FHIT* gene may occur earlier than K-*ras* or *APC* mutation. Furthermore, our results raise the possibility that the inactivation of *FHIT* may contribute to the development of colorectal tumors in concert with mutation of *APC* in early stage. Hao *et al.* (8) recently reported that the frequency of reduced expression of Fhit protein was 11 of 55 cases (20%) in human colon adenomas and 44% of 84 cases in adenocarcinomas as judged only by immunohistochemistry. Western blot analysis performed in the present study proved to be a more sensitive for detection of altered FHIT expression than immunohistochemistry.

There are some reports that Fhit functions as a tumor suppressor gene, such as *RB* or *p53*. Siprashvili *et al.* (7) showed that replacement of the wild-type *FHIT* gene in human cancer cell lines that lacked endogeneous Fhit expression significantly reduced their tumorigenicity in nude mice. Recently, it was reported that Fhit expression by adenovirus vector reduced the transplanted tumor burden and acted on the induction of apoptosis (17). In contrast, Otterson *et al.* (18) reported that introduction of wild-type *FHIT* into HeLa cells, a cervical cancer cell line, did not alter its tumorigenecity in animals. In our case, the exogeneous expression of human Fhit protein in human colon cancer cells SW480 caused a marked inhibition of growth and rendered SW480 cells highly susceptible to induction of apoptosis compared with control cells. Moreover, the number of SW/FHIT colonies in soft agar was significantly fewer than that of SW/IRES colonies (data not shown). Fhit protein seems to use an apoptotic pathway to execute its tumor suppression activity, however, the growth suppression and induction of apoptosis by Fhit may depend on the specificity of the cell lineage. Recently, it was reported that all of the *Fhit*+/− mice developed multiple tumors of the stomach and skin tumors similar to those observed in a human cancer syndrome. Muir-Torre syndrome, which is caused by deficiency in a mismatch repair gene (19). Taken together, our results raised the possibility that *FHIT* mutation may induce a genetic instability leading to another abnormality of tumor-associated genes. It remains to be elucidated how Fhit protein exerts its tumor suppression activity in normal cells.

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REFERENCES

1. Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Moir, M., McCue, P., Druck, T., Croce, C. M., and Huebner, K. (1996) *Cell* **84,** 587–597.
2. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranonowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M., and Huebner, K. (1996) *Biochemistry* **35,** 11529–11535.
3. Sozzi, G., Veronese, M. L., Negrini, M., Baffa, R., Cotticelli, M. G., Inoue, H., Tornielli, S., Pilotti, S., DeGregorio, L., Pastorino, U., Pierotti, M. A., Ohta, M., Huebner, K., and Croce, C. M. (1996) *Cell* **85,** 17–26.
4. Gemma, A., Hagiwara, K., Ke, Y., Burke, L. M., Khan, M. A., Nagashima, M., Bennet, W. P., and Harris, C. C. (1997) *Cancer Res.* **57,** 1435–1437.
5. Virgilio, L., Shuster, M., Gollin, S. M., Veronese, M. L., Ohta, M., Huebner, K., and Croce, C. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9770–9775.
6. Sozzi, G., Sard, L., DeGregorio, L., Marchetti, A., Musso, K., Buttitta, F., Tornielli, S., Pellegrini, S., Veronese, M. L., Manenti, G., Incarbone, M., Chella, A., Angeletti, C. A., Pastorino, U., Huebner, K., Bevilaqua, G., Pilotti, S., Croce, C. M., and Pilotti, M. A. (1997) *Cancer Res.* **57,** 2121–2123.
7. Siprashvili, Z., Sozzi, G., Barnes, L. D., MuCue, P., Robinson, A. K., Eryomin, V., Sard, L., Tagliabue, E., Greco, A., Fusetti, L., Schwartz, G., Pierotti, M. A., Croce, C. M., and Huebner, K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 13771–13776.
8. Hao, X. P., Willis, J. E., Pretlow, T. G., Rao, J. S., MacLennan, G. T., Talbot, I. C., and Pretlow, T. P. (2000) *Cancer Res.* **60,** 18–21.
9. Markwell, M. A. K., Hass, S. M., Tolbert, N. E., and Bieber, L. L. (1981) *in* Methods in Enzymology (Lowenstein, M. L., Ed.), pp. 296–303, Academic Press, New York.
10. Akao, Y., Mizoguchi, H., Ohishi, N., and Yagi, K. (1998) *FEBS Lett.* **429,** 279–183.
11. Mancuso, T., Aguilar, F., Pescarolo, M. P., Clerico, L., Russo, P., and Parodi, S. (1997) *Nucleic Acids Res.* **25,** 3643–3648.
12. Evans, C. H., and DiPaolo, J. A. (1975) *Cancer Res.* **35,** 1035–1044.
13. Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Ham-

ilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. (1992) *Nature* **359,** 235–337.

14. Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., Peterson, G., Hamilton, S. R., Kinzler, K. W., Vogelstein, B., and Nakamura, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4452–4456.
15. Muto, T., Nagawa, H., Watanabe, T., Masaki, T., and Sawada, T. (1997) *Dis. Colon Rectum* **40**(Suppl), 580–585.
16. Croce, C. M., Sazzi, G., and Huebner, K. (1999) *J. Cli. Oncol.* **17,** 1618–1624.
17. Ji, L., Fang, B., Yen, N., Fong, K., Minna, J. D., and Roth, J. A. (1999) *Cancer Res.* **59,** 3333–3339.
18. Otterson, G. A., Xiano, G. H., Geradts, J., Jin, F., Chen, W. D., Niklinska, W., Kaye, F. J., and Yeung, R. S. (1998) *J. Natl. Cancer Inst.* **90,** 426–432.
19. Fong, L. Y. Y., Fidanza, V., Zanesi, N., Lock, L. F., Siracusa, L. D., Mancini, R., Siprashvili, Z., Ottey, M., Martin, S. E., Druck, T., McCue, P. A., Croce, C. M., and Huebner, K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4742–4747.