[CANCER RESEARCH 62, 6820–6822, December 1, 2002]

Advances in Brief

Aberrant Methylation of the *HPP1* Gene in Ulcerative Colitis-associated Colorectal Carcinoma[1]

Fumiaki Sato,[2] David Shibata,[2] Noam Harpaz, Yan Xu, Jing Yin, Yuriko Mori, Suna Wang, Andreea Olaru, Elena Deacu, Florin M. Selaru, Martha C. Kimos, Prodromos Hytiroglou, Joanne Young, Barbara Leggett, Adi F. Gazdar, Shinichi Toyooka, John M. Abraham, and Stephen J. Meltzer[3]

Gastroenterology Division, Department of Medicine, University of Maryland School of Medicine and Gastroenterology Service, Department of Medicine, Baltimore VA Hospital, Baltimore, Maryland 21201 [F. S., Y. X., J. Yi., Y. M., S. W., A. O., E. D., F. M. S., M. C. K., J. M. A., S. J. M.]; Division of Surgical Oncology, Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland 21201 [D. S.]; Department of Pathology, The Mount Sinai School of Medicine, New York, New York 10029 [N. H., P. H.]; Gastroenterology Unit, Queensland Institute of Medical Research, Brisbane 4029, Australia [J. Yo., B. L.]; and University of Texas Southwestern Medical Center, Hamon Cancer Center, Dallas, Texas 75390 [A. F. G., S. T.]

Abstract

The *HPP1* gene was cloned as a frequently methylated gene in hyperplastic polyps of the colon. It has been shown that *HPP1* expression is silenced by *HPP1* gene hypermethylation in sporadic colorectal cancers. To determine the role of *HPP1* in ulcerative colitis (UC)-associated carcinogenesis, the prevalence of *HPP1* methylation was investigated in three different histological stages of UC-associated carcinogenesis (non-neoplastic UC colon, dysplasia, and carcinoma). Quantitative methylation-specific PCR and quantitative reverse transcription-PCR were used to determine *HPP1* gene methylation and expression levels, respectively. *HPP1* methylation was observed in 24 of 48 (50%) adenocarcinomas and in 4 of 10 (40%) dysplasias. In contrast, no non-neoplastic UC mucosa showed *HPP1* methylation. *HPP1* expression in the HCT116 colon cancer cell line was restored after treatment with the demethylating agent 5-aza-2′-deoxycytidine. In conclusion, our data suggest that methylation of *HPP1* is a relatively common early event in UC-associated carcinogenesis. *HPP1* offers potential as a biomarker for the early detection of cancer or dysplasia in UC.

Introduction

UC[4] is a chronic disease characterized by inflammation of the mucosa and submucosa of the large intestine. The duration and extent to which a patient suffers from UC correlate directly with an increased propensity to develop colorectal carcinoma (1, 2). For patients who have had UC for more than 20 years, the incidence of colorectal cancer is 10–20-fold greater than that of the general population, and the average age of onset is 20 years earlier (3). UC-associated colorectal carcinoma is different from sporadic carcinoma: unlike sporadic colorectal carcinoma, which arises from adenomatous polyps, UC-associated colorectal carcinoma progresses from areas of dysplastic mucosa. Although the molecular events that facilitate the progression of adenoma to carcinoma in sporadic colorectal cancer have been well investigated (4), much remains to be learned regarding molecular events underlying the progression of UC mucosa to dysplasia and carcinoma.

Adenomas are the precursors of most sporadic colorectal cancers. Using a strategy that isolates differentially methylated sequences from hyperplastic polyps and normal mucosa, Young *et al.* (5) identified a 370-bp sequence containing the 5′-untranslated region and the first exon of a gene encoding a transmembrane protein. This gene was named *HPP1* and noted to contain two follistatin modules and an EGF-like domain. By RT-PCR, *HPP1* was found to be expressed in 28 of 30 (93%) normal colonic samples but in only 7 of 30 (23%) colorectal cancers. The 5′ region of *HPP1* included a CpG island containing 49 CpG sites, 96% of which were methylated in colonic tumor.

To determine whether *HPP1* hypermethylation occurs during the progression of UC mucosa to carcinoma, the frequency and timing of *HPP1* hypermethylation were investigated in clinical samples ranging from non-neoplastic UC mucosa to colorectal carcinoma.

Materials and Methods

Tissue Samples. Fifty-eight tumor tissues and five non-neoplastic UC mucosae were obtained at the time of surgical resection from 47 patients with one or more UC-associated colorectal neoplasms. The UC-associated neoplasms consisted of 48 adenocarcinomas and 10 dysplasias. All tissues were grossly dissected free of normal surrounding tissue, and parallel sections were used for histological characterization. Although microdissection was not performed, the tissues were selected to include only those tumors containing >70% tumor cells by H&E staining.

DNA and RNA Extraction. Genomic normal and tumor DNAs were extracted using previously published protocols (6, 7). RNAs were extracted by Trizol reagent (Invitrogen).

Quantitative MSP. DNA methylation of *HPP1* was determined by quantitative MSP (8) using the Taqman system. MSP distinguishes methylated alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers and probe specific to the corresponding methylated DNA sequences is then performed. Primers and probe for quantitative MSP were designed using the GenBank AF264150 sequence for *HPP1*. Primer and probe sequences for *HPP1* are listed in Table 1. *β-Actin* was selected as an internal control, and analysis was performed using previously published primer and probe sequences (8). Briefly, 0.5 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol, and resuspended in 50 μl of water. The PCR mixture consisted of 12.5 μl of Taqman Universal Master Mix without UNG (Applied Biosystems, CA), 0.25 μl of each forward and reverse primer of both *HPP1* and *β-Actin* (10 μM), 2.0 μl of probe for both *HPP1* and *β-Actin* (2.5 μM), 50 mg of bisulfite-treated DNA, and H_2O (up to 25 μl in total volume). PCR reaction and real-time data collection were performed using an ABI7700 Sequence Detection System (Applied Biosystems, CA) for activation of Taq polymerase at 95°C for 10 min and 50 cycles consisting of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. CpGenome Universal Methylated DNA (Intergen) was used to generate a standard curve for each reaction. Reaction mix without any bisulfite-treated DNA was used as a negative control. MSP values were calculated using the formula below.

$$\text{MSP value} = (\textit{HPP1-S}/\textit{HPP1-T})/(\textit{β-Actin-S}/\textit{β-Actin-T})$$

Received 5/2/02; accepted 10/18/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by USPHS Grants DK47717, CA95323, CA85069, and CA 77057 and Early Detection Research Network Grant 5U01CA8497102 (National Cancer Institute, Bethesda, MD).

[2] These authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at University of Maryland School of Medicine, Room N3W62, 22 South Greene Street, Baltimore, MD 21201. Phone: (410) 706-3375; Fax: (410) 706-1325; E-mail: smeltzer@medicine.umaryland.edu.

[4] The abbreviations used are: UC, ulcerative colitis; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; 5-Aza-dC, 5-aza-2′-deoxycytidine; EGF, epidermal growth factor.

Table 1 *Sequences of primers and probes for HPP1 gene*

Primers and probes	Sequences (5′→3′)
Quantitative MSP	
Forward	GTTATCGTCGTCGTTTTTGTTGTC
Reverse	GACTTCCGAAAAACACAAAATCG
Probe	6FAM-CCGAACAACGAACTACTAAACATCCCGCG-TAMRA
Quantitative RT-PCR	
Forward	TGCTTTCCCTACCTCCTTAAGTGA
Reverse	CTGTCATCATAACCAGAGCAATTCC
Probe	6FAM-TGCCAAACGCCCACCGGC-TAMRA

HPP1-S and *HPP1-T* represent levels of *HPP1* methylation in the sample and totally methylated control DNAs, respectively. *β-Actin-S* and *β-Actin-T* correspond to amplified *β-Actin* in the sample and totally methylated DNAs, respectively. Because the *β-Actin* primer and probe sequences do not contain any CpGs, *β-Actin* amplification is unaffected by methylation status. Consequently, the quantitative MSP value is representative of the percentage of sample DNA that is densely methylated.

Quantitative RT-PCR. The expression level of the *HPP1* gene was measured using the Taqman quantitative RT-PCR system. The primer and probe sequences for *HPP1* are listed in Table 1. The reverse primer was designed to overlap the exon 1–2 boundary of the *HPP1* mRNA sequence to avoid amplification of potentially contaminated genomic DNA. cDNA from samples was synthesized by the conversion of 2 μg of total RNA using the SuperScript II kit (Invitrogen) and random primers (N_6), according to the manufacturer's recommendations. cDNA generated from 500 ng of tRNA was used for each reaction. The PCR mixture consisted of 12.5 μl of Taqman Universal MasterMix with UNG (Applied Biosystems, CA): 0.25 μl of each forward and reverse primer (10 μM), and probe (2.5 μM) of both *HPP1* and *β-Actin*, cDNA, and H_2O (up to 25 μl in total volume). The Taqman RNase control reagent (VIC dye-labeled; Applied Biosystems) was used for normalization of data. A standard curve was generated in each experiment by cDNA from an unpublished gastric fibroblast cell strain that highly expressed *HPP1* mRNA. Ratio to this standard sample represented the relative expression level of *HPP1* mRNA.

5-Aza-dC Treatment of Colon Cancer Cell Line HCT116. HCT116 (American Type Culture Collection) is a colorectal cancer cell line that expresses very low levels of *HPP1* by quantitative RT-PCR and in which the *HPP1* gene is highly methylated as determined by quantitative MSP. The 5-Aza-dC (Sigma) treatment procedure has been published previously (9). Briefly, cells (1×10^5) were seeded in a 100-mm dish. Twenty-four h later, cells were treated with 10^{-6} M 5-Aza-dC for 24 h. The media were changed at the end of the treatment and once for 3 days. DNA and RNA were obtained at 2, 4, and 6 days after the treatment.

Results

Quantitative MSP and RT-PCR of *HPP1* in Colon Cancer Cell Line HCT116. *HPP1* demethylation by 5-Aza-dC and the associated restoration of *HPP1* expression are illustrated in Fig. 1. The *HPP1* gene in HCT116 was highly methylated before 5-Aza-dC treatment. However, the level of methylation was gradually diminished after 5-Aza-dC treatment. This was associated with a concomitant increase in the level of *HPP1* mRNA expression.

Quantitative MSP and RT-PCR of *HPP1* in Clinical Tissues. An inverse relationship between *HPP1* methylation and expression levels is suggested by the analysis of eight tumors and one dysplastic tissue specimen (Fig. 2). All three samples in which the *HPP1* MSP value was <0.1 exhibited high *HPP1* mRNA expression. Therefore, we designated a MSP value of 0.1 as a cutoff point to assign negative or positive methylation status. Using this limit, five of six methylation-positive samples expressed low levels of *HPP1* mRNA.

Quantitative MSP of *HPP1* in UC-associated Colorectal Cancers. The *HPP1* methylation status of UC-associated colorectal tumors is displayed in Fig. 3. Using a MSP value of 0.1 as the determinant, aberrant methylation of *HPP1* was observed in 4 of 10 (40%) dysplasias and 24 of 48 (50%) carcinomas, whereas no methylation was detected in 5 non-neoplastic UC mucosae.

Discussion

The purpose of this study was to determine the frequency and timing of *HPP1* hypermethylation in UC-associated colorectal carcinogenesis. In this study, hypermethylation of *HPP1* was present in 4 of 10 (40%) dysplasias and in 24 of 48 (50%) carcinomas. Using conventional MSP, Young *et al.* (5) reported that the frequency of *HPP1* hypermethylation is 63% in hyperplastic colonic polyps, 66% in colonic adenomas, and 84% in sporadic colorectal cancers. In comparison with these data, our *HPP1* hypermethylation frequencies are relatively lower. In our study, we used quantitative PCR techniques for both MSP and RT-PCR. Using a MSP value of 0.1 as a dichotomization point may exclude low levels of methylation (1:100 to 1:1000 diluted totally methylated DNA) that could produce visible bands by conventional MSP (10, 11). This may potentially explain the differences in *HPP1* hypermethylation prevalence between sporadic colorectal and UC-associated neoplasms.

HPP1 aberrant methylation was observed in the early stages of

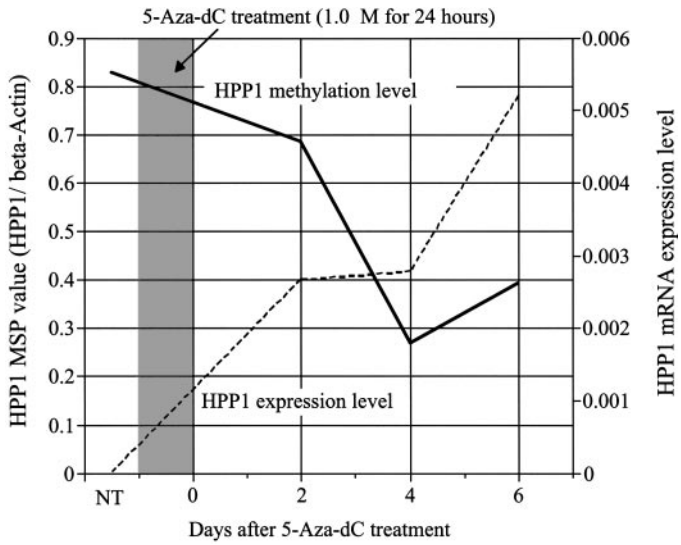

Fig. 1. Effect of 5-Aza-dC treatment on demethylation of the *HPP1* gene and restoration of *HPP1* expression. The *solid line* represents *HPP1* MSP values (*left Y* axis), and the *dashed line* represents *HPP1* expression levels (*right Y* axis), whereas the *gray area* indicates the timing of 5-Aza-dC treatment. The *HPP1* RT-PCR value was expressed as a relative expression ratio to a reference sample, which was used to generate a standard curve. *HPP1* methylation levels increased between days 4 and 6 after removal of 5-Aza-dC, possibly due to a growth advantage for methylated cells.

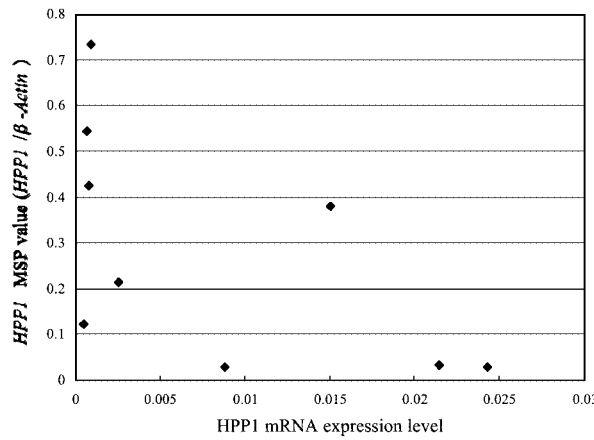

Fig. 2. Relationship of *HPP1* methylation and expression levels in UC-associated clinical tissues. *HPP1* methylation and expression levels of UC-associated colorectal tumor and one dysplasia are displayed in a scatter plot format (*X* axis, *HPP1* mRNA expression level; *Y* axis, *HPP1* methylation level).

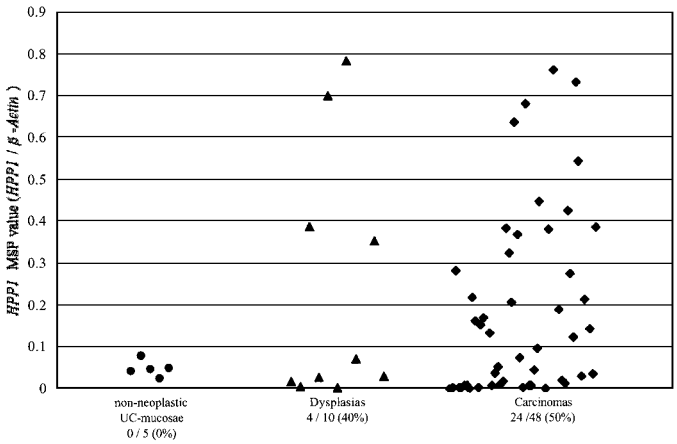

Fig. 3. Prevalence of *HPP1* methylation in UC-associated colorectal neoplasms. *HPP1* methylation rate in each group was estimated using a MSP value cutoff point of 0.1.

UC-associated carcinogenesis and is similar to what is observed during the development of sporadic colorectal cancers. Aberrant methylation of age- and cancer-related genes (*e.g.*, *estrogen receptor*, $p16^{INK4a}$, *E-cadherin*, *hMLH1*, $p14^{ARF}$, and so forth) has been reported in the setting of UC-associated dysplasia and colorectal cancers (11–15). Furthermore, hypermethylation of tumor suppressor genes has been reported in other precancerous lesions associated with chronic inflammation [*e.g.*, *E-cadherin* in chronic gastritis and *APC*, $p16^{INK4a}$, and *E-cadherin* in Barrett's esophagus (16, 17)]. Therefore, a chronic inflammatory status may predispose to the early onset of aberrant methylation of multiple genes.

In this study, we demonstrated that *HPP1* expression was restored in the colon cancer cell line HCT116 by 5-Aza-dC treatment. Young *et al.* (5) have also demonstrated that *HPP1* expression was restored in two colon cancer cell lines (HT29 and Lovo) by 5-Aza-dC treatment. Recently, Liang *et al.* (18) demonstrated differential gene expression between 5-Aza-dC-treated cells and nontreated cells. In this microarray-based study, some genes were up-regulated, whereas others were down-regulated by 5-Aza-dC treatment. This would suggest that changes in gene expression caused by 5-Aza-dC may reflect a balance between inducible negative and positive transcriptional factors. In our temporal analysis of methylation and expression, *HPP1* expression was up-regulated on day 6 in the face of a slightly elevated methylation level. Some unknown positive regulators of *HPP1* may have affected the expression of *HPP1* at this particular time point. Hypermethylation may represent a major regulatory process in the expression of *HPP1*.

In our study, we encountered one clinical sample that exhibited relatively high *HPP1* mRNA expression in the setting of positive methylation. This finding may be explained by the fact that *HPP1* is expressed not only in epithelial cells but also in myofibroblasts (5). Variable levels of contamination by myofibroblasts could have resulted in this paradoxical observation. Alternatively, due to the competing influences of methylation *versus* inducible transcription factors, methylation of the *HPP1* locus may not consistently correlate with complete silencing of *HPP1* mRNA expression.

The HPP1 molecule has both a transmembranous form and a soluble form, with one EGF module and two follistatin modules in the extracellular domain and a potential G protein-activating motif in the cytoplasmic domain (19). This suggests that HPP1 may be a multifunctional protein and that it could play roles as both a growth factor and a receptor. HPP1 stimulates tyrosine phosphorylation of erbB4 (EGFR4) in the gastric cancer cell line MKN28 (19) and can prolong the survival of neural cells (20). However, the significance of HPP1 function in the gastrointestinal tract remains unclear.

In conclusion, our data suggest that aberrant methylation of *HPP1* is a relatively common and early event in UC-associated carcinogenesis. *HPP1* is worthy of further study to elucidate its biological function and also to explore its potential as a biomarker for the early detection of cancer or dysplasia in UC.

References

1. Lashner, B. A., Silverstein, M. D., and Hanauer, S. B. Hazard rates for dysplasia and cancer in ulcerative colitis. Results from a surveillance program. Dig. Dis. Sci. *34:* 1536–1541, 1989.
2. Ekbom, A., Helmick, C., Zack, M., and Adami, H. O. Ulcerative colitis and colorectal cancer. A population-based study. N. Engl. J. Med., *323:* 1228–1233, 1990.
3. Harpaz, N., and Talbot, I. C. Colorectal cancer in idiopathic inflammatory bowel disease. Semin. Diagn. Pathol., *13:* 339–357, 1996.
4. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61:* 759–767, 1990.
5. Young, J., Biden, K. G., Simms, L. A., Huggard, P., Karamatic, R., Eyre, H. J., Sutherland, G. R., Herath, N., Barker, M., Anderson, G. J., Fitzpatrick, D. R., Ramm, G. A., Jass, J. R., and Leggett, B. A. *HPP1*: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. Proc. Natl. Acad. Sci. USA, *98:* 265–270, 2001.
6. Meltzer, S. J., Yin, J., Manin, B., Rhyu, M. G., Cottrell, J., Hudson, E., Redd, J. L., Krasna, M. J., Abraham, J. M., and Reid, B. J. Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. Cancer Res., *54:* 3379–3382, 1994.
7. Boynton, R. F., Blount, P. L., Yin, J., Brown, V. L., Huang, Y., Tong, Y., McDaniel, T., Newkirk, C., Resau, J. H., Raskind, W. H., *et al.* Loss of heterozygosity involving the APC and MCC genetic loci occurs in the majority of human esophageal cancers. Proc. Natl. Acad. Sci. USA, *89:* 3385–3388, 1992.
8. Eads, C. A., Lord, R. V., Wickramasinghe, K., Long, T. I., Kurumboor, S. K., Bernstein, L., Peters, J. H., DeMeester, S. R., DeMeester, T. R., Skinner, K. A., and Laird, P. W. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res., *61:* 3410–3418, 2001.
9. Bender, C. M., Gonzalgo, M. L., Gonzales, F. A., Nguyen, C. T., Robertson, K. D., and Jones, P. A. Roles of cell division and gene transcription in the methylation of CpG islands. Mol. Cell. Biol., *19:* 6690–6698, 1999.
10. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
11. Sato, F., Harpaz, N., Shibata, D., Xu, Y., Yin, J., Mori, Y., Zou, T. T., Wang, S., Desai, K., Leytin, A., Selaru, F. M., Abraham, J. M., and Meltzer, S. J. Hypermethylation of the $p14^{ARF}$ gene in ulcerative colitis-associated colorectal carcinogenesis. Cancer Res., *62:* 1148–1151, 2002.
12. Issa, J. P., Ahuja, N., Toyota, M., Bronner, M. P., and Brentnall, T. A. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res., *61:* 3573–3577, 2001.
13. Hsieh, C. J., Klump, B., Holzmann, K., Borchard, F., Gregor, M., and Porschen, R. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. Cancer Res., *58:* 3942–3945, 1998.
14. Wheeler, J. M., Kim, H. C., Efstathiou, J. A., Ilyas, M., Mortensen, N. J., and Bodmer, W. F. Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. Gut, *48:* 367–371, 2001.
15. Fleisher, A. S., Esteller, M., Harpaz, N., Leytin, A., Rashid, A., Xu, Y., Liang, J., Stine, O. C., Yin, J., Zou, T. T., Abraham, J. M., Kong, D., Wilson, K. T., James, S. P., Herman, J. G., and Meltzer, S. J. Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1. Cancer Res., *60:* 4864–4868, 2000.
16. Tamura, G., Yin, J., Wang, S., Fleisher, A. S., Zou, T., Abraham, J. M., Kong, D., Smolinski, K. N., Wilson, K. T., James, S. P., Silverberg, S. G., Nishizuka, S., Terashima, M., Motoyama, T., and Meltzer, S. J. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. J. Natl. Cancer Inst. (Bethesda), *92:* 569–573, 2000.
17. Eads, C. A., Lord, R. V., Kurumboor, S. K., Wickramasinghe, K., Skinner, M. L., Long, T. I., Peters, J. H., DeMeester, T. R., Danenberg, K. D., Danenberg, P. V., Laird, P. W., and Skinner, K. A. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res., *60:* 5021–5026, 2000.
18. Liang, G., Gonzales, F. A., Jones, P. A., Orntoft, T. F., and Thykjaer, T. Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2′-deoxycytidine. Cancer Res., *62:* 961–966, 2002.
19. Uchida, T., Wada, K., Akamatsu, T., Yonezawa, M., Noguchi, H., Mizoguchi, A., Kasuga, M., and Sakamoto, C. A novel epidermal growth factor-like molecule containing two follistatin modules stimulates tyrosine phosphorylation of erbB-4 in MKN28 gastric cancer cells. Biochem. Biophys. Res. Commun., *266:* 593–602, 1999.
20. Horie, M., Mitsumoto, Y., Kyushiki, H., Kanemoto, N., Watanabe, A., Taniguchi, Y., Nishino, N., Okamoto, T., Kondo, M., Mori, T., Noguchi, K., Nakamura, Y., Takahashi, E., and Tanigami, A. Identification and characterization of TMEFF2, a novel survival factor for hippocampal and mesencephalic neurons. Genomics, *67:* 146–152, 2000.

Aberrant Methylation of the *HPP1* Gene in Ulcerative Colitis-associated Colorectal Carcinoma

Fumiaki Sato, David Shibata, Noam Harpaz, et al.

Cancer Res 2002;62:6820-6822.