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Advances in Brief

Hypermethylation of *HPP1* Is Associated with *hMLH1* Hypermethylation in Gastric Adenocarcinomas[1]

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Abstract

The *HPP1* gene was initially discovered because of its frequent hypermethylation in hyperplastic colon polyps, but it is also hypermethylated in colorectal adenomas and carcinomas. Expression of the DNA mismatch repair gene *hMLH1* is diminished or absent in some hyperplastic polyps, and it has been suggested that *HPP1* inactivation is associated with the progression of microsatellite-unstable colorectal tumors. We sought then to determine the prevalence of *HPP1* silencing by DNA methylation in gastric adenocarcinomas and to define any association of this event with microsatellite instability (MSI) or *hMLH1* hypermethylation. Thirty-two matched normal-gastric adenocarcinoma DNA pairs were studied for MSI status and hypermethylation of *HPP1* and *hMLH1*. Five (100%) of 5 MSI-H tumors, 2 (50%) of 4 MSI-L tumors, and 8 (35%) of 23 MSS tumors demonstrated *HPP1* hypermethylation. Eight (25%) of 32 tumors (5 of 5 MSI-H, 2 of 4 MSI-L, and 1 of 23 MSS) showed evidence of *hMLH1* hypermethylation. All (8 of 8) of these hMLHI-methylated tumors demonstrated concomitant methylation at the *HPP1* locus: there were no cases of *hMLH1* methylation occurring in the absence of *HPP1* methylation. In gastric adenocarcinoma, hypermethylation frequently targets *HPP1*. Moreover, *hMLH1* hypermethylation occurs predominantly in the setting of *HPP1* hypermethylation. *HPP1* hypermethylation may represent an early event in mismatch repair-deficient gastric tumorigenesis.

Introduction

In various tumor types, a growing number of genes have been recognized as undergoing aberrant CpG island methylation, which is associated with transcriptional repression and loss of gene function (1). Using a global methylation screening assay to isolate differentially methylated sequences in hyperplastic polyps from patients with hyperplastic polyposis, Young *et al.* (2) previously discovered a 370-bp sequence containing the 5′ untranslated region and the first exon of a novel gene called *HPP1*.

HPP1 is predicted to encode a cell surface receptor with a short cytoplasmic tail, a transmembrane domain and an extracellular component with two follistatin modules, an epidermal growth factor-like domain, a phosphorylation site, and a binding site for glycosaminoglycans (2). It demonstrates a high degree of structural homology with tomoregulin, which is restricted in its distribution to neurons and glial cells of the central nervous system but also to pericryptal myofibroblasts in the gastrointestinal tract (3). *HPP1* also shares homology with a gene called *TMEFF2*, which may play a role in regulating the survival of hippocampal and mesencephalic neurons (4). Pericryptal myofibroblasts are thought to be paracrine cells responsible for coordinating epithelial proliferation, differentiation, and apoptosis (5). The exact function of *HPP1* has not been elucidated; however, like tomoregulin, its epidermal growth factor-like domain appears to be a ligand for c-erbB-4, whereas its follistatin domains may bind and regulate transforming growth factor β (2). It is therefore possible that *HPP1* may play multiple roles in cell growth, maturation, and adhesion, and its inactivation may serve as an early event in the initiation of gastrointestinal neoplastic progression.

The role of adenomas as precursors of sporadic MSI[4]-high colorectal cancers has been questioned. Hyperplastic polyps have been linked to sporadic colorectal cancers with MSI and may, in fact, represent precursor lesions for this particular subset of colon carcinomas (6). In the only study of *HPP1* hypermethylation published to date, *HPP1* hypermethylation occurred in the majority of colorectal adenomas, hyperplastic polyps, and colorectal cancers (2). The occurrence of *HPP1* methylation across a range of colorectal neoplasms implies that *HPP1* alteration may not be the primary initiating event in hyperplastic polyp formation. However, transcriptional suppression of *HPP1* may contribute to the development of a subset of hyperplastic polyps which, in turn, serve as precursors of microsatellite-unstable colorectal cancers (6).

Another type of cancer that is characterized by frequent MSI is sporadic gastric cancer (7). As in colorectal cancer, MSI in sporadic gastric cancer is most frequently caused by hypermethylation of the *hMLH1* gene promoter (8–10). In the current study, we determined the prevalence of *HPP1* hypermethylation in gastric adenocarcinomas and defined associations of this event with both MSI and *hMLH1* hypermethylation.

Materials and Methods

Cell Lines. The gastric adenocarcinoma cell lines AGS, SIIA, and MKN28 were used in this study. AGS was obtained from American Type Culture Collection (Manassas, VA). Cells were propagated in appropriate cell culture media.

Primary Tumor Samples. Thirty-two matched normal and gastric adenocarcinoma pairs were obtained at the time of surgical resection and were freshly frozen. Genomic normal and tumor DNAs were extracted using standard protocols (11). RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications.

Real-Time MSP. Real-time MSP (12) using TaqMan technology was performed using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). 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 uracil. Subsequent PCR using primers and probe specific to sequences corresponding to methylated DNA se-

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[4] The abbreviations used are: MSI, microsatellite instability; MSP, methylation-specific PCR; 5AzaDC, 5-Aza-2′-deoxycytidine.

quences is then performed. Primers and probe sequences for *HPP1* were designed using the GenBank AF264150 sequence (5′ to 3′ F: GTTATCGTCGTCGTTTT-TGTTGTC, R: GACTTCCGAAAAACACAAAATCG, and Probe: 6FAM-CCGAACAACGAACTACTAAACATCCCGCG-TAMRA). Published primer and probe sequences were used for *hMLH1* and the internal control, *β-actin* (12). (*hMLH1*: 5′ to 3′, F: TAATCTATCGCCGCCTCATCG, R: TCGTTATATAT-CGTTCG-TAGTATTCGTGTTTAGTTTC, and Probe: 6FAM-CGAACGCGA-CGTCAAACGCCACT-A-TAMRA).

Briefly, 0.5 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified with the Wizard DNA Purification Resin (Promega, Madison, WI), retreated with NaOH, precipitated with ethanol, and resuspended in 50 μl of water.

PCR amplification was performed using a 96-well optical tray with a final reaction mixture of 25 μl consisting of 12.5 μl of TaqMan Universal Mastermix without uracil DNA glycosylase (Applied Biosystems, Foster City, CA), 0.25 μl of respective forward and reverse primers (10 μM) for either *HPP1* or *hMLH1*, 0.25 μl of forward and reverse primers (10 μM) for β-actin, 2 μl of probe for either *HPP1* or *hMLH1* (2.5 μM), 2 μl of probe for β-actin (2.5 μM), and 50 ng of bisulfite-modified DNA and water. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. CpGenome Universal Methylated DNA (Intergen, Purchase, NY) was used to generate a standard curve for each reaction. Reaction mix without any bisulfite-modified DNA was used as a negative control.

Real-Time RT-PCR. *HPP1* gene expression was measured by TaqMan-based real-time reverse transcriptase-PCR. Primer and probe sequences are as follows: 5′ to 3′, F: TGCTTTCCCTACCTCCTTAAGTGA, R: CTGTCAT-CATAACCAGAGCAATTCC, and Probe: 6FAM-TGCCAAACGCCCACC-G-GC-TAMRA. The reverse primer was designed to overlap the exon 1–2 boundary of the *HPP1* sequence to avoid amplification of contaminated genomic DNA. Two μg of total RNA were converted to cDNA using the SuperScipt II kit (Invitrogen) and random primers (N_6) according to the manufacturer's recommendations.

Reverse transcriptase-PCR amplification was performed using a 96-well optical tray with a final reaction mixture of 25 μl consisting of 12.5 μl of TaqMan Universal Mastermix with uracil DNA glycosylase (Applied Biosystems), 0.25 μl of forward and reverse *HPP1* primers (10 μM), 2 μl of *HPP1* probe (2.5 μM), cDNA generated from 500 ng of total RNA and water. A VIC-dye-labeled, TaqMan RNase control reagent (Applied Biosystems) was used for normalization of data. Standard curves were generated using cDNA from an unpublished gastric fibroblast cell strain, which expressed high levels of *HPP1* mRNA. Ratio to this standard sample represented the relative expression level of *HPP1* mRNA.

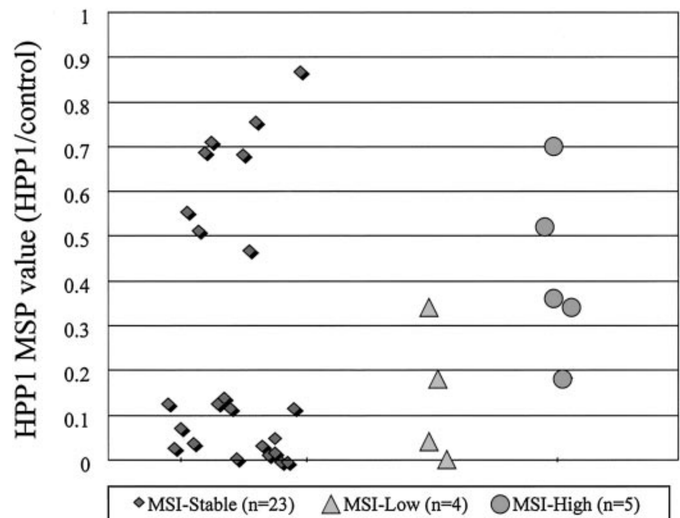

Fig. 2. *HPP1* hypermethylation in primary gastric adenocarcinomas. A total of 15 (47%) of 32 gastric cancers demonstrated *HPP1* gene hypermethylation. The difference in the prevalence of *HPP1* methylation between microsatellite unstable (MSI-L and MSI-H) tumors and stable (MSS) tumors was statistically significant ($P < 0.05$) by Fisher's exact test.

Analysis of Real-Time PCR. For real-time MSP, a MSP value was calculated by dividing the ratio of Gene:*β-actin* for a sample by the Gene:*β-actin* ratio for Universal Methylated DNA. All samples for which the MSP value was <0.15 exhibited high mRNA expression for either *HPP1* or *hMLH1*. An MSP value of 0.15 was designated as the cutoff point for classifying a result as positive (≥0.15) or negative (<0.15) for methylation.

Statistics. Comparisons between methylation prevalences were performed using Fisher's Exact Test.

MSI Status. The MSI status of each tumor was determined using previously published methods using five microsatellite loci (*D2S123*, *D5S346*, *D17S250*, *BAT25*, and *BAT26*; Ref. 13). Tumors were then categorized as either MSI-High (MSI-H; ≥2 loci), MSI-Low (MSI-L; 1 locus), and MSI-stable (MSS; no loci).

5-Aza-2′-Deoxycytidine (5AzaDC) Treatment. AGS cells were seeded at 1×10^5 cells/ml in a 100-mm culture dish using appropriate culture media. Twenty-four h later, cells were treated with 0.1 μM 5AzaDC for a 24-h period. Media were changed at the end of treatment and then at 3 days after treatment. DNA and RNA were extracted from cells at pretreatment, immediately after treatment, and at days 2, 4, and 6.

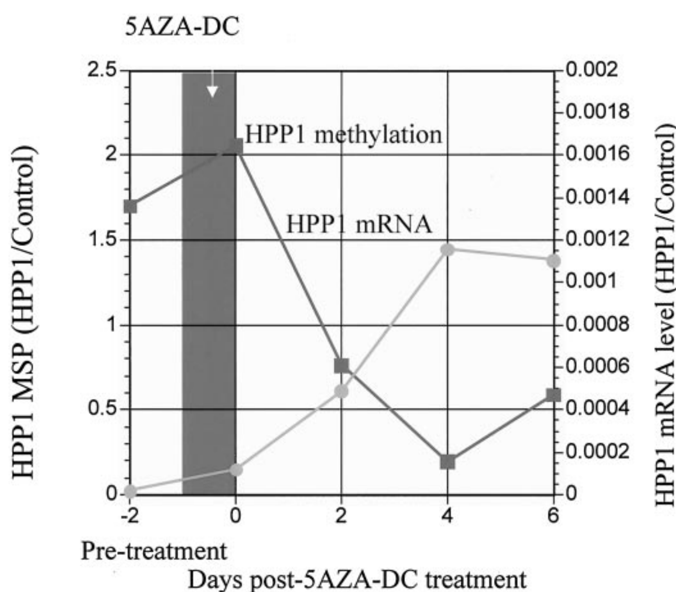

Fig. 1. Effects of 5AzaDC on *HPP1* methylation and expression in the AGS gastric cancer cell line. 5AzaDC treatment results in temporally progressive *HPP1* demethylation and is associated with re-expression of *HPP1*. Maximal demethylation and *HPP1* re-expression were noted at day 4 after 5AzaDC treatment.

Results

Real-Time MSP and Reverse Transcriptase-PCR Analysis of *HPP1* in Gastric Cancer Cell Lines. *HPP1* hypermethylation and diminished mRNA expression were demonstrated in the AGS and SIIA cell lines but not in MKN28 cells. The AGS cell line exhibited the highest level of *HPP1* methylation and was selected for treatment with 5-AzaDC. A direct and temporal correlation was demonstrated between *HPP1* methylation and mRNA expression (Fig. 1).

MSI Status. After MSI analysis, 5 tumors were classified as MSI-H, 4 as MSI-L, and 23 as MSS.

Analysis of *HPP1 Methylation*. Using real-time MSP, we demonstrated that *HPP1* gene hypermethylation was present in 15 (47%) of 32 gastric cancers. Five (100%) of 5 MSI-H, 2 (50%) of 4 MSI-L tumors and 8 (35%) of 23 MSS tumors demonstrated *HPP1* hypermethylation (Fig. 2). In addition, one normal mucosal specimen (of 32) from a patient with a methylation-positive MSS tumor was hypermethylated at *HPP1*.

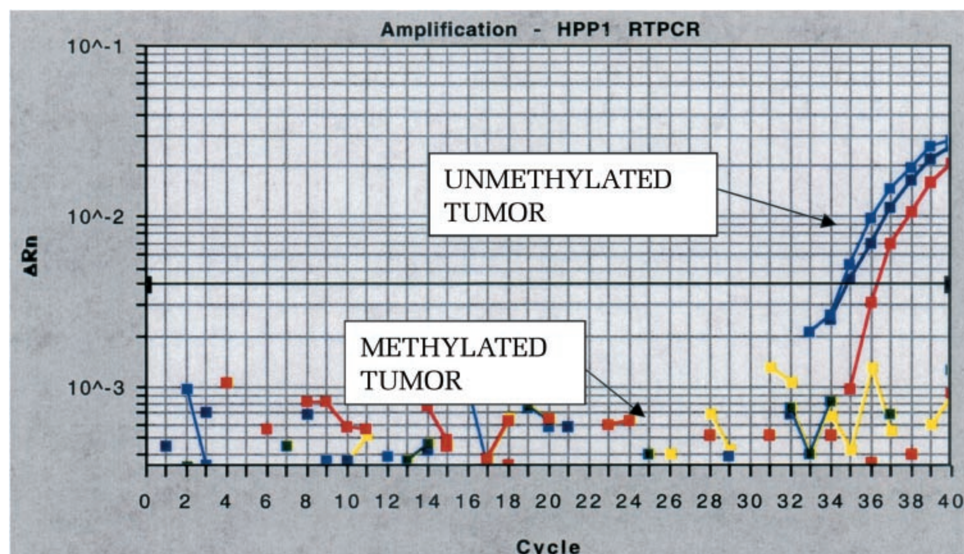

Fig. 3. *HPP1* mRNA expression in gastric cancers with and without *HPP1* methylation. A representative real-time reverse transcriptase-PCR amplification plot is depicted comparing a *HPP1* methylated tumor to an unmethylated tumor. *HPP1* expression is present in the unmethylated tumor (—— tracing) while it is suppressed in the methylated tumor (—— tracing). Analyses were performed in triplicate.

HPP1 mRNA Expression in Gastric Tumor Samples. *HPP1* mRNA expression was assessed in four selected gastric cancers from both *HPP1*-methylated and unmethylated categories. *HPP1* mRNA was expressed in *HPP1*-unmethylated tumors but was attenuated in methylated tumors (Fig. 3).

Analysis of *hMLH1* Hypermethylation. Eight (25%) of 32 tumors (5 of 5 MSI-H, 2 of 4 MSI-L, and 1 of 23 MSS) showed evidence of *hMLH1* hypermethylation. One of 32 normal mucosal specimens exhibited *hMLH1* hypermethylation.

Correlation Between *HPP1* and *hMLH1* Hypermethylation. Three different subsets of gastric cancers were identified: (*a*) both *HPP1* and *hMLH1* methylation status low or negative; (*b*) *HPP1* gene methylated but *hMLH1* methylation negative; and (*c*) both genes hypermethylated (Fig. 4). One corresponding normal mucosal specimen from a patient with an MSS gastric tumor was found to have hypermethylation at both the *HPP1* and *hMLH1* loci. All (8 of 8) *hMLH1*-hypermethylated tumors demonstrated concomitant methylation at the *HPP1* locus. There were no cases in which *hMLH1* was methylation-positive and *HPP1* was methylation-negative.

Discussion

In this study, *HPP1* hypermethylation occurred in approximately half of the gastric cancers analyzed. When stratified by MSI status, these data revealed a statistically significant association of *HPP1* hypermethylation with MSI (occurring in 78% of MSI-H and MSI-L tumors *versus* 35% of MSS tumors). Perhaps the most striking finding in this study was that *hMLH1* hypermethylation was exclusively associated with *HPP1* hypermethylation: *i.e.,* there were no cases of *hMLH1* hypermethylation occurring in the absence of *HPP1* hypermethylation.

Young *et al.* (2) found that *HPP1* methylation occurs in 84% of colorectal cancers. Contrary to our findings, they did not note any significant difference in *HPP1* methylation when tumors were stratified by MSI status. In their study, significant *HPP1* methylation occurred infrequently in normal tissue, mostly in patients with MSI-H tumors (2). Similarly, in our analysis, only 1 of 32 normal gastric mucosal specimens showed evidence of *HPP1* hypermethylation. This single normal specimen was paired with a gastric cancer classified as microsatellite-stable but, nevertheless, demonstrated concomitant methylation of *hMLH1* and *HPP1*. Hypermethylation of *hMLH1* was not specifically studied in the experiments of Young *et al.* (2). Our MSI classification was based on national consensus recommendations of analyzing five microsatellite loci (14). However, evaluation of a larger number of loci could have lead to reclassification of the MSI status in our tumors, including the one with *HPP1* methylation in matching normal tissue.

It has been postulated that colorectal adenomas do not represent the progenitors of sporadic MSI-H colorectal cancers (2). Although MSI is relatively rare in sporadic colorectal cancers and adenomas, Jass *et al.* (15) reported MSI in a large percentage of colon cancers arising in patients with hyperplastic polyposis. This finding suggests a possible association between hyperplastic polyps and MSI-H colorectal carcinogenesis. Jass *et al.* (15) have proposed the serrated adenoma as the intermediate lesion in a discrete pathway leading to the development of MSI-H colorectal cancers. Given the high prevalence of *HPP1* methylation in hyperplastic polyps, adenomas, and carcinomas, it is unlikely that this epigenetic event is unique to the development of hyperplastic polyps. Nevertheless,

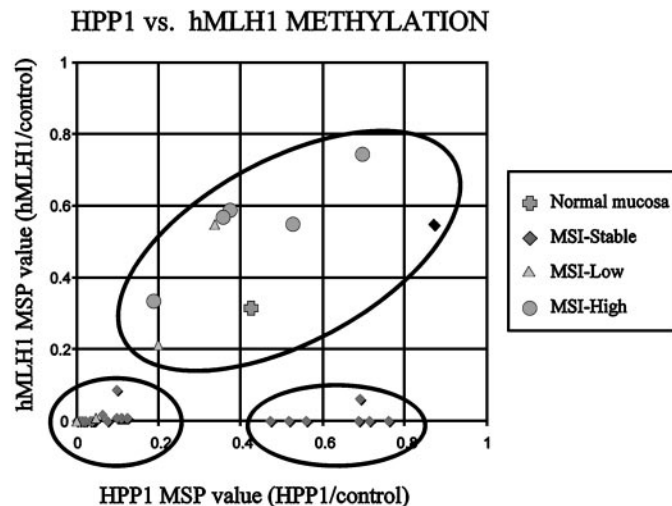

Fig. 4. Relationship between *HPP1* and *hMLH1* methylation status in gastric adenocarcinoma. Three subsets of gastric cancers were identified. The first group demonstrated low or no methylation of both *HPP1* and *hMLH1* (*lower left oval*). A second group was found to have *HPP1* methylation but not *hMLH1* methylation (*lower right oval*). The final group was found to have methylation of both genes (*large upper oval*). One normal mucosal specimen with hypermethylation of both genes was included in the third group.

epigenetic silencing of *HPP1* may underlie a subset of hyperplastic polyps which, after subsequent inactivation of *hMLH1*, develop into sporadic microsatellite-unstable tumors.

Precursor lesions of gastric adenocarcinoma have not been as well characterized as their counterparts in colorectal carcinogenesis. However, gastric cancer shares with colorectal cancer a relatively high frequency of MSI (7). *HPP1* hypermethylation may represent an early event in the evolution of gastric neoplasia that precedes *hMLH1* hypermethylation and is required for entry into the pathway leading to microsatellite-unstable gastric cancer. Alternatively, hypermethylation of these two genes may be a related process, with hypermethylation of *HPP1* being a more frequent event.

Additional studies of the MSI-H subset of gastric tumors may reveal additional methylation events supporting the existence of an aberrant methylator phenotype in gastric oncogenesis. A precedent for this theory exists in the association between CpG Island Methylator Phenotype or CIMP and MSI, which has been reported in colorectal cancers (16, 17). Elucidation of such a pathway in gastric cancers may permit targeted analysis of putative precursor lesions, such as gastric adenomas and dysplastic lesions, in order to predict malignant potential and prognosis.

We conclude that in gastric adenocarcinomas, hypermethylation frequently targets *HPP1* and correlates with its transcriptional silencing. Moreover, *hMLH1* hypermethylation occurs predominantly in the setting of *HPP1* hypermethylation. *HPP1* inactivation in gastric oncogenesis may represent an early event in a pathway that culminates in disordered DNA mismatch repair.

References

1. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. A gene hypermethylation profile of human cancer. Cancer Res., *61:* 3225–3229, 2001.
2. 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.
3. 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.
4. 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.
5. Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I., and West, A. B. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. Am. J. Physiol., *277:* C183–C201, 1999.
6. Jass, J. R., Young, J., and Leggett, B. A. Hyperplastic polyps and DNA microsatellite unstable cancers of the colorectum. Histopathology, *37:* 295–301, 2000.
7. Rhyu, M. G., Park, W. S., and Meltzer, S. J. Microsatellite instability occurs frequently in human gastric carcinoma. Oncogene, *9:* 29–32, 1994.
8. Bevilacqua, R. A., and Simpson, A. J. Methylation of the *hMLH1* promoter but no *hMLH1* mutations in sporadic gastric carcinomas with high-level microsatellite instability. Int. J. Cancer, *87:* 200–203, 2000.
9. Fleisher, A. S., Esteller, M., Wang, S., Tamura, G., Suzuki, H., Yin, J., Zou, T. T., Abraham, J. M., Kong, D., Smolinski, K. N., Shi, Y. Q., Rhyu, M. G., Powell, S. M., James, S. P., Wilson, K. T., Herman, J. G., and Meltzer, S. J. Hypermethylation of the *hMLH1* gene promoter in human gastric cancers with microsatellite instability. Cancer Res., *59:* 1090–1095, 1999.
10. Wheeler, J. M., Loukola, A., Aaltonen, L. A., Mortensen, N. J., and Bodmer, W. F. The role of hypermethylation of the *hMLH1* promoter region in HNPCC *versus* MSI+ sporadic colorectal cancers. J. Med. Genet., *37:* 588–592, 2000.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
12. Eads, C. A., Danenberg, K. D., Kawakami, K., Saltz, L. B., Blake, C., Shibata, D., Danenberg, P. V., and Laird, P. W. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res., *28:* E32, 2000.
13. Mori, Y., Yin, J., Rashid, A., Leggett, B. A., Young, J., Simms, L., Kuehl, P. M., Langenberg, P., Meltzer, S. J., and Stine, O. C. Instabilotyping: comprehensive identification of frameshift mutations caused by coding region microsatellite instability. Cancer Res., *61:* 6046–6049, 2001.
14. Boland, C. R., Thibodeau, S. N., Hamilton, S. R., Sidransky, D., Eshleman, J. R., Burt, R. W., Meltzer, S. J., Rodriguez-Bigas, M. A., Fodde, R., Ranzani, G. N., and Srivastava, S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res., *58:* 5248–5257, 1998.
15. Jass, J. R. Hyperplastic polyps of the colorectum-innocent or guilty? Dis. Colon Rectum, *44:* 163–166, 2001.
16. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. CpG island methylator phenotype in colorectal cancer. Proc. Natl. Acad. Sci. USA, *96:* 8681–8686, 1999.
17. Ahuja, N., Mohan, A. L., Li, Q., Stolker, J. M., Herman, J. G., Hamilton, S. R., Baylin, S. B., and Issa, J. P. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res., *57:* 3370–3374, 1997.