Hypermethylation of HPP1 Is Associated with hMLH1 Hypermethylation in Gastric Adenocarcinomas

David M. Shibata, Fumiaki Sato, Yuriko Mori, Kellie Perry, Jing Yin, Suna Wang, Yan Xu, Andreea Olaru, Florin Selaru, Kevin Spring, Joanne Young, John M. Abraham, and Stephen J. Meltzer

Division of Surgical Oncology, Department of Surgery [D. M. S., K. P.] and Division of Gastroenterology, Department of Medicine [F. Sa., Y. M., K. Yi., S. W., Y. X., A. O., F. Se., J. M. A., S. J. M.] University of Maryland School of Medicine, Baltimore, Maryland 21201, and Conjoint Gastroenterology Unit, Queensland Institute of Medical Research, Brisbane 4029, Australia [K. S., J. Yo.]

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 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-nine 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 hMLH1-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). Pericytchal 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.

Another type of cancer that is characterized by frequent MSI is sporadic gastric cancer (7). 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, SIA, 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.

Received 7/11/02; accepted 9/3/02.

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 This work was supported in part by United States Public Health Service Grants DK74717, CA95523, CA95069, and CA77057. These authors contributed equally to this work.

2 These authors contributed equally to this work.

To whom requests for reprints should be addressed, at University of Maryland School of Medicine 22 South Greene Street, Room NJW62, Baltimore, MD 21201, Phone: (410) 706-3375, Fax: (410) 706-1325; E-mail: Smeltzer@medicine.umd.edu.

4 The abbreviations used are: MSI, microsatellite instability; MSP, methylation-specific PCR; 5AzA DC, 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: GTTATCCGTCCTTGGTTGTC, R: GACCTCGAAACACAAATCTG, and Probe: 6FAM-CGCAAGACGAACTAAACTTAATCAG-3′). Published primer and probe sequences were used for *hMLH1* and the internal control, β-actin (12).


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: TGTCTTCCCTACTCCTTATACTG, R: CGCTCATCATAACGGAGCAAATCTCC, and Probe: 6FAM-TGCAAAAGCCACCCAG-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 SuperScript II kit (Invitrogen) and random primers (N6) 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.

**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 × 10⁵ 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.

**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. 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.

**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.
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. 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.](#)

![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
Hypermethylation of HPP1 Is Associated with hMLH1 Hypermethylation in Gastric Adenocarcinomas

David M. Shibata, Fumiaki Sato, Yuriko Mori, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/20/5637

Cited articles
This article cites 14 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/20/5637.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/62/20/5637.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.