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 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 cancer 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 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 ng of bisulfite-treated DNA, and 1.5 mM MgCl₂ (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. CpgMeth 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.

\[
\text{MSP value} = \left( \frac{HPP1}{\text{β-Actin}} \right) \times \frac{1}{\left( \frac{\text{β-Actin}}{\text{β-Actin}} \right)}
\]
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 (N₆), 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 H₂O (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 x 10⁵) 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. 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...
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\textsuperscript{INK4a}, E-cadherin, hMLH1, p14\textsuperscript{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\textsuperscript{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). Levels of contamination by myofibroblasts could have resulted in this paradoxic 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

Aberrant Methylation of the \textit{HPP1} Gene in Ulcerative Colitis-associated Colorectal Carcinoma

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