HPP1: A transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers

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Adenomas are the precursors of most colorectal cancers. Hyperplastic polyps have been linked to the subset of colorectal cancers showing DNA microsatellite instability, but little is known of their underlying genetic etiology. Using a strategy that isolates differentially methylated sequences from hyperplastic polyps and normal mucosa, we identified a 370-bp sequence containing the 5′ untranslated region and the first exon of a gene that we have called HPP1. Rapid amplification of cDNA ends was used to isolate HPP1 from normal mucosa. Using reverse transcription–PCR, HPP1 was expressed in 28 of 30 (93%) normal colonic samples but in only seven of 30 (23%) colorectal cancers (P < 0.001). The 5′ region of HPP1 included a CpG island containing 49 CpG sites, of which 96% were found to be methylated by bisulfite sequencing of DNA from colonic tumor samples. By COBRA analysis, methylation was detected in six of nine (66%) adenomas, 17 of 27 (63%) hyperplastic polyps, and 46 of 55 (84%) colorectal cancers. There was an inverse relationship between methylation level and mRNA expression in cancers (r = −0.67; P < 0.001), and 5-aza-2-deoxycytidine treatment restored HPP1 expression in two colorectal cancer cell lines. In situ hybridization of HPP1 indicated that expression occurs in epithelial and stromal elements in normal mucosa but is silenced in both cell types in early colonic neoplasia. HPP1 is predicted to encode a transmembrane protein containing follistatin and epidermal growth factor-like domains. Silencing of HPP1 by methylation may increase the probability of neoplastic transformation.

The evolution of colorectal cancer occurs as a stepwise process described at the morphological level as the adenoma–carcinoma sequence. Inherited colorectal cancer syndromes have yielded fundamental insights into the underlying molecular mechanisms. In familial adenomatous polyposis, inactivation of the “gatekeeper” APC gene initiates the development of multiple adenomas by disrupting growth control. Most sporadic colorectal neoplasms are believed to be initiated by a similar mechanism, and subsequent progression occurs through the development of chromosomal instability. In hereditary nonpolyposis colorectal cancer, adenoma progression is accelerated through the early inactivation of a “caretaker” DNA mismatch repair gene and establishment of DNA instability (1). A deficiency of DNA mismatch repair is also implicated in the subset of sporadic colorectal cancers showing DNA microsatellite instability (MSI); the usual underlying mechanism is silencing of the DNA mismatch repair gene hMLH1 by promoter methylation (2). However, sporadic adenomas rarely show either MSI (3) or loss of expression of DNA mismatch repair genes except in the context of hereditary nonpolyposis colorectal cancer (3, 4). Moreover, the frequency of APC mutation is reduced in sporadic MSI cancers (5, 6). These observations cast doubt on the role of adenoma as the precursor of sporadic MSI-high colorectal cancer.

Hyperplastic polyps are less well characterized than adenomas but are related epidemiologically to colorectal cancers (7). In normal colorectal mucosa, epithelial cells mature as they migrate along the crypt column and are lost from the surface epithelium (8). In hyperplastic polyps, there is delayed cell migration, and hypermature cells are retained within the surface epithelium (9). These observations suggest a primary defect in migration or exfoliation, but the mechanism is unknown. The risk of colorectal cancer is increased in subjects developing multiple hyperplastic polyps (10, 11), and hyperplastic polyps may show clonal genetic alterations that are also described in colorectal cancer. These include K-ras mutations (12), allele loss on chromosome 1p (13), and transforming growth factor βRII mutations (14). K-ras may be an initiating mutation in small hyperplastic polyps (13, 15). However, the mechanism initiating large multiple and proximally located hyperplastic polyps, which have been linked to cancers showing DNA MSI (13, 16, 17), is unknown.

A methylator phenotype in which some promoter sequences are hypermethylated recently has been shown to be associated with a subset of colorectal cancers, notably cancers with MSI (18, 19). Because hyperplastic polyps may show MSI, methylation may have had a role in their initiation. Using a strategy that isolates differentially methylated sequences from colonic neoplasms and corresponding normal colonic mucosa, we identified a sequence of 370 bp containing the 5′-untranslated region and the first exon of a novel gene, which we have called HPP1, because it initially was identified in subjects with hyperplastic polyposis. This report describes the isolation and characterization of HPP1.

Materials and Methods

Samples. This study was carried out on a subset of 55 sporadic colorectal cancers from a series of 303 previously characterized according to their level of DNA MSI (20). Of these, 19 were high-level MSI, 18 were low-level MSI, and 18 were microsatellite stable. Also included were 10 adenomas and 27 hyperplastic polyps freshly collected from patients with hyperplastic polyposis. DNA was extracted from all samples by a salt precipitation technique (21).

Global Methylation Screening Assay. Paired normal and neoplastic DNA samples from individuals with hyperplastic polyposis and
cancer were digested with \( RsaI \) to create smaller fragments and further digested with restriction enzymes having differential sensitivities to cytosine methylation (\( MspI \) and \( HpaII \)), before undergoing arbitrarily primed PCR amplification (ref. 22; Fig. 1a) in the presence of \( 33P \)-dATP. Fragments were resolved on sequencing gels, and those showing differential methylation were eluted from the gel, cloned into pGEM-T vectors (Promega), and sequenced. Fragment sequences underwent BLAST searches against GenBank and European Molecular Biology Laboratory databases.

**Confirmation of Methylation.** Methylation of CpG sites within \( HPP1 \) was confirmed by using bisulfite sequencing (23) and nested primer sets (\( HPBisF \) and \( HPBisR \) as the outer set and \( HPBisF2 \) and \( HPBisR2 \) as the inner set) in nine putatively methylated and five unmethylated DNA samples analyzed by arbitrarily primed (AP)-PCR. To screen larger numbers of samples for \( HPP1 \)-specific methylation, we used a COBRA assay (24) to produce restriction fragments from bisulfite-treated DNA only if methylation was present. We used the primers described above to amplify fragments of 427 bp, which were cleaved into fragments of 271 and 156 bp by digestion with \( RsaI \). Cleavage fragments were quantified by densitometry. Results of digestion by \( RsaI \) correlated with the results of bisulfite sequencing of the CpG site that formed part of the \( RsaI \) recognition sequence in all 14 samples analyzed by AP-PCR, as well as CpG dinucleotides in the surrounding DNA. A control digest was performed with \( AluI \), which digested DNA that had not undergone bisulfite modification, and this was used to check for incomplete conversion. \( HPP1 \) COBRA assays were performed on 55 sporadic colorectal cancers, 10 adenomatous polyps, and 27 hyperplastic polyps as well as corresponding normal mucosa samples.

**Sequencing of Full-Length cDNA and Rapid Amplification of cDNA Ends (RACE).** cDNA was synthesized from 3 \( mg \) total RNA from pooled normal colonic mucosa using Superscript II (GIBCO/BRL) reverse transcriptase and random hexamers. A 506-bp cDNA fragment spanning a portion of the 5' untranslated region (UTR) was amplified with a downstream primer within the methylated fragment (\( HPX \)) and an upstream primer designed from an extended region of homologous database sequence (\( HPA \)). Similarly, a 781-bp cDNA fragment was produced from an upstream primer within the methylated fragment (\( HPV \)) and a downstream primer in the coding portion of homologous database sequence (\( HPV \)). A 581-bp cDNA fragment was produced from an upstream primer within the methylated fragment (\( HPC \)) and a downstream primer in the coding portion of homologous database sequence (\( HPC \)). These overlapping fragments were sequenced and checked for homology to database entries. In addition, a further
828-bp fragment was sequenced, which contained the cDNA equivalent portion of the methylated fragment (HPA and HPW 5'-TGACTAGTTTCTCCAGGCTTCCAT-3'). Then, 3' RACE was performed with human colonic mucosa 3'-RACE-Ready cDNA and human colorectal cancer 3'-RACE-Ready cDNA (CLONTECH). The template for 3' RACE was derived from either five pooled normal mucosa total RNA samples or seven pooled tumor total RNA samples known to express HPP1 by reverse transcription–PCR (RT-PCR). Amplification was carried out with the sense primer NGSP2 (5'-TTGAGCAT-TCTCTGATAATTCTGTTCTTGC-3') and an antisense modified oligo(dT) primer, Advantage 2 polymerase (CLONTECH), and a touchdown PCR program. PCR products were excised from agarose gels and subcloned into pGEM-T vectors (Promega) for sequencing.

**RT-PCR.** RT-PCR of HPP1 was performed on a subset of cases in which fresh frozen tissue was available (30 cancers, 1 adenoma, and 1 hyperplastic polyp) and on which COBRA assays had already been performed. RNA was extracted from paired normal and tumor tissues using RNA Isolation Reagent (ABgene) according to the manufacturer's recommendations. cDNA was synthesized as described above. Primers were designed (HPC and HPZ 5'-TGAACGAGACGCAAGGCAC-3') that amplified a 306-bp fragment spanning a portion of the 5' UTR and coding sequence of HPP1. cDNA products were resolved on 2% agarose gels and stained briefly in 0.5 µg/ml ethidium bromide. Loading was controlled by the simultaneous PCR of β-actin cDNA (25). RT-PCR primers spanned an intron of HPP1 and hence were unlikely to amplify genomic DNA.

**Manipulation of HPP1 Expression by 5-Aza-2-Deoxycytidine (5-AzaC).** HT29 adenocarcinoma (microsatellite stable) cells and Lovo (high-level MSI) cells (American Type Culture Collection) are two colorectal cancer lines that do not express HPP1 by RT-PCR, and in which HPP1 DNA is 100% methylated by COBRA assay. The demethylating agent 5-AzaC (Sigma) was used to reverse the methylation-suppressed expression of HPP1 (26). HT29 and Lovo were seeded at 1 x 10⁶ cells/ml in 10 ml of RPMI and exposed to concentrations of 5-AzaC ranging from 0 to 10.0 µM after 48 h of growth. Cells were harvested after 72 h of exposure, and RNA was extracted. A final concentration of 0.5 µM 5-AzaC produced optimal induction of HPP1 expression without cytoxicity. Both lines were again seeded as described above to study the timing of HPP1 expression induction. Cells were grown in 0.5 µM with 5-AzaC for 72 h and then incubated without 5-AzaC for a further 72 h. Cells were harvested immediately before and at 12-h intervals after the initial contact with 5-AzaC. All cells harvested underwent RT-PCR for HPP1.

**In Situ Hybridization (ISH) of HPP1 mRNA.** For detection of HPP1 mRNA in paraffin-embedded tissue, an 828-bp fragment of HPP1 cDNA was subcloned into a PGEM-T vector. A 300-bp fragment for ISH was produced by alkaline hydrolysis. Digoxigenin-labeled riboprobes, for sense (control) and antisense, were produced for HPP1 by in vitro transcription with SP6 and T7 polymerases as described (27). ISH was performed on 5-μm sections, deparaffinized by xylol, and rehydrated by gradient alcohol before exposure to hydrochloric acid (0.2 mol/liter), as described (28). Sections were permeabilized with 5 mg/ml proteinase K at 37°C for 15 min, followed by fixation in 4% paraformaldehyde for 20 min at room temperature. Prehybridization (50% formamide/1% SDS/5× standard saline citrate/500 mg/ml RNA/50 mg/ml heparin) was performed at 67.5°C for 4 h followed by hybridization for 16 h at 67.5°C in a solution containing 1 mg/ml digoxigenin-labeled riboprobe. Sections were washed of unbound probe and incubated with alkaline phosphatase-conjugated antidigoxigenin polyclonal sera (1:200) for 2 h. Unbound antibody was removed by washes, followed by visualization with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate for 16 h. Unbound complex was removed by washing, and sections were counterstained with eosin. ISH was performed on 15 colorectal cancers (six expressing HPP1 by RT-PCR, six in which no expression was seen, and three with no RT-PCR results), one adenoma, and six hyperplastic polyps as well as 18 normal colonic mucosa samples.

**Immunochemistry of α Smooth Muscle Actin (SMA).** Intestinal sections were incubated with a mouse monoclonal antibody to α-SMA (1:400; Dako), followed by a biotinylated rabbit anti-mouse IgG as the secondary antibody, as previously described. The detection system used was a Dako streptavidin–biotin complex/horseradish peroxidase kit, with 3,3-diaminobenzidine tetrahydrochloride as the chromogenic substrate. Sections were counterstained with eosin (27).

**Loss of Heterozygosity (LOH) Studies.** Coding region primers (HPC and HPZ) were used to find the boundary between exons 1 and 2 by comparing the sequences obtained after amplification of both genomic and cDNA. Sequencing of this boundary in genomic DNA revealed a CT repeat in intron 1 (GenBank accession no. AF264150). The CT repeat was amplified with primers designed to produce a fragment of 244 bp (HPC 5'- GCTGATTCCTGGCAAAGGTGCC-3' and HPZ 5'-CT-CAACTCCCTGTACTACCTTG-3') in 50 blood donor controls and in paired samples of normal and tumor DNA. Allele loss was analyzed in 55 colorectal cancers, 10 adenomatous polyps, and 27 hyperplastic polyps as described (17).

**Chromosomal Localization of HPP1.** The cDNA fragment amplified by the primers HPA and HPW was cloned into a pGEM-T vector (Promega) and used to find the chromosomal location of HPP1 by fluorescence ISH. The probe was nick-translated with biotinylated dATP and hybridized in situ at a final concentration of 20 ng/µl to metaphases from two normal males. The fluorescence ISH method was modified from that previously described (29) in that chromosomes were stained before analysis with both propidium iodide and 4',6-diamidino-2-phenylindole. Images of metaphase preparations were captured by a cooled charge-coupled device camera using the chromoscan image collection and enhancement system (Applied Imaging, Newcastle, U.K.).

**Results**

**Global Methylation Assay and Identification of HPP1.** Using a screening assay on a small selected group of samples, several fragments were found to have differential methylation in both colorectal polyps and cancers. The most frequently methylated of these fragments is shown in Fig. 1b and was methylated in both polyps and cancers but only rarely in normal mucosa. The methylated fragment was 370 bp in length and contained a dense cluster of 49 CpG sites, which spanned the 5' UTR and the first exon of a gene we have called HPP1, as it was initially isolated from a patient with hyperplastic polyposis (GenBank accession no. AF264150; Fig. 1c). The cluster qualified as a CpG island under standard criteria (30). Sequencing of overlapping cDNA fragments spanning a portion of the 5' UTR and 755 bp of coding sequence, which included the methylated fragment, revealed that the fragment showed greater than 95% homology to six other cDNA sequences [TMP-2 (GenBank accession no. E14369), tomoregulin (GenBank accession no. AB004064), TMEFF2 (GenBank accession no. ABO17269), DKFZp564L1878 (GenBank accession no. HSM082469), TPEF (GenBank accession nos. AF242222 and AF242221; ref. 31), and TENB2 (GenBank accession no. AF179274)]. Analysis of the translated sequences revealed follistatin domains and a trans-
membrane region as described (GenBank accession no. AAD55776).

RACE. We used 3' RACE to identify the balance of the gene; this resulted in the isolation of two coding region variants of HPP1 (Fig. 1d). The most frequently isolated clone was HPP1-A, which would be predicted to produce a full-length transcript with 95% homology to TENB2. The coding region of the less frequently isolated form HPP1-B was homologous to DKFZp564L1878, a clone containing a 57-bp insertion after nucleotide 1028 with three STOP signals (GenBank accession no. HSM802469; Fig. 1e). These signals would be predicted to truncate the protein just short of the cytoplasmic tail domain. Both variants were found in pooled normal mucosa, whereas in pooled HPP1 expressing cancer, only HPP1-A was observed. Variations in the 3' UTR were seen in forms isolated from both normal and tumor. Clones homologous to the tomoregulin 3'-coding region were not isolated.

Confirmation of HPP1 as a Methylation Target. Bisulfite sequencing was performed in the 5' UTR of HPP1 in 2-5 clones each of a subset of neoplastic samples (six colorectal cancers, three hyperplastic polyps), which showed methylation by the global methylation assay, and five normal mucosal samples, which were negative by this assay (Fig. 2e). The fragment sequenced included 44 CpG sites and was densely methylated. In those nine DNA samples in which methylation was detected by AP-PCR, an average of 96% of CpG sites was methylated per clone. All clones were methylated at the RsaI site used in the COBRA assay. The COBRA assay was used to confirm HPP1-specific methylation (Fig. 2b). Methylation levels of >30% were observed in 46 of 55 (84%) colorectal cancers, six of nine (66%) adenomas, and 17 of 27 (63%) hyperplastic polyps. When cancers were analyzed with respect to MSI status, 17 of 19 (89%) MSI-high, 13 of 18 (72%) MSI-low, and 16 of 18 (88%) microsatellite stable tumors showed >30% methylation. Methylation occurred at low levels (less than 30%) in 48 of 55 (87%) normal mucosa samples. Seven normal samples showed methylation levels of >30%, and five of these occurred in patients with an MSI-high tumor (P = 0.041, Fisher's Exact test; Fig. 2b). Analysis of COBRA values in normal mucosa as a continuous variable showed no significant correlation with age, sex, or site of sampling in the colon. In addition, there was no correlation between levels of HPP1 methylation in cancers and size, site, or MSI status of the tumor or age and sex of the patient, whether COBRA levels were analyzed as a continuous or discrete variable. Exposure to the demethylating agent 5-AzaC restored the expression of HPP1 in the two colorectal cancer cell lines. HPP1 expression was observed 12 h after initial treatment and was still present 72 h after the demethylating agent was removed (Fig. 2d).

RT-PCR and Correlation with COBRA. HPP1 was expressed in 28 of 30 (93%) normal mucosa samples but in only seven of 30 (23%) corresponding colorectal cancers tested (Fig. 2c). This represents a significant decrease in expression in cancers (P = <0.001, Fisher's Exact test). Cancers lost expression uniformly across MSI classes. A significant correlation was found between the level of methylation and the expression of HPP1 (r = 0.67, P <
Expression of HPP1 was observed in the pericyrtal myofibroblasts and other stromal cells of normal colon mucosa, and in epithelium where it was accentuated in terminally differentiated cells of the upper crypts. Expression also was seen in B-lymphocytes in gut-associated lymphoid tissue, scattered fibroblasts in the submucosa, ganglion cells, and endothelial cells (Fig. 3a and b). Small hyperplastic polyps (n = 4) retained the patterns of expression seen in normal mucosa. In larger hyperplastic polyps (n = 2) and the single adenoma, HPP1 was not expressed in epithelium and stroma but was detected in lymphoid aggregates (Fig. 3c and d). Junctions between normal mucosa and polyp showed a transition zone adjacent to the polyp in which expression gradually was extinguished. In cancers negative for HPP1 by RT-PCR (n = 6), there was only trace expression with the exception of one sample that showed moderate expression. In the cancers expressing HPP1 by both RT-PCR and ISH (n = 6), expression by ISH was more obvious in epithelium than stroma, although the stroma remained weakly positive.

Immunohistochemistry of αSMA. The pericyrtal myofibroblasts are strap-like cells running close to and parallel with the crypt vertical axis. Unlike other stromal cells, these expressed HPP1 as well as SMA. Cells that expressed SMA but not HPP1 included smooth muscle cells in muscularis mucosae and vessel walls. Cancer and polyp myofibroblasts were also shown to express SMA, indicating that these cells were still represented in neoplastic lesions when HPP1 expression was abolished (Fig. 3e).

Chromosomal Localization of HPP1. Twenty metaphases from the first normal male were examined for fluorescent signal. All of these metaphases showed signal on one or both chromatids of chromosome 2 in the region 2q32-q33 (Fig. 4a). There was a total of 11 nonspecific background dots observed in the 20 metaphases. A similar result was obtained from hybridization with HPP1 to 10 metaphases from the second normal male (data not shown).

LOH Studies. The first exon of HPP1 forms a boundary with exon 2 after 174 bp of coding sequence. Within the intervening intron, there is an imperfect CT repeat of 23 units interrupted by a single AT after CT number 15 (GenBank accession no. AF264150). This microsatellite (CT23i) was found to be a 14-allele polymorphism with heterozygosity of 0.76 when tested on 50 unrelated Caucasian blood donors. LOH was observed in one of 22 (5%) colorectal cancers, three of six (50%) adenomas, and none of 22 hyperplastic polyps that were informative at CT23i (Fig. 4b). The single carcinoma in which LOH at HPP1 was observed was a sporadic, proximal, poorly differentiated signet ring cancer in a 67-yr-old female. The three adenomas that showed LOH at HPP1 all were derived from a 56-yr-old female with multiple adenomata. Both patients also demonstrated methylation of HPP1 in their neoplasms.

Discussion

Although the adenoma has long been recognized as the principal precancerous lesion in the colorectum, evidence is accumulating that the hyperplastic polyp may be the precursor of a subset of colorectal cancers. Neoplastic evolution has been well documented in hyperplastic polyposis (10, 11, 13), and previous work from our laboratory has shown that hyperplastic polyps are linked to the microsatellite unstable pathway of carcinogenesis (16). Because MSI is associated with the hypermethylation phenotype (18), it is possible that the mechanism underlying the pathway of serrated neoplasia would be uncovered through the demonstration of differential methylation. Accordingly, we studied normal, hyperplastic, and malignant colorectal epithelium with a global methylation analysis technique and identified a gene HPP1.

We have demonstrated that the expression of HPP1 can be induced by the treatment of nonexpressing cell lines with a demethylating agent. A high degree of correlation was seen between the level of methylation and expression of HPP1 by RT-PCR; however, some discordant values were recorded. In five samples, high levels of methylation were accompanied by significant expression of HPP1. In these cases, it is possible that the expression may originate from stromal elements (see Fig. 3c). In a further five samples, there was no apparent methylation despite loss of expression, which may indicate the presence of an inactivating mutation leading to transcript instability, another form of transcriptional
silencing, or an upstream mutational event. Alternatively, discordant values could be explained by atypical site-specific methylation or demethylation events at the single CpG dinucleotide probed in the COBRA assay (32). Allelic loss was not a common mechanism of inactivation of HPP1.

TMP-2, tomoragulin, TMEM27 TPEF, and TENB2, which all show a high degree of homology to HPP1, are predicted to be transmembrane proteins containing follistatin, glycosaminoglycan binding, and epidermal growth factor-like domains. The expression of tomoragulin in adult tissues is apparently restricted to neurons and glial cells of the central nervous system and fibroblasts in the gastrointestinal mucosa (33), although we were unable to isolate tomoragulin from pooled normal mucosa by 3’ RACE. In view of its putative role as a ligand for er-B4 and its follistatin domains, it has been suggested that tomoragulin could be implicated in the coordination of proliferative differentiation, or apoptosis of gastrointestinal tissues (33). Follistatin domains have been identified in other proteins such as osteonectin. Osteonectin (SPARC) is found in the lens of the eye and functions as an anti-adhesive disrupting cellular contact with matrix (34). When SPARC is rendered nonfunctional in the mouse, cataracts develop, and disordered maturation and migration of cells is observed (35). A molecule with homology to SPARC is predicted to underlie the normal development of gut mucosa (36), and platelet-derived growth factor A- and B-deficient mice show a high degree of homology to SPARC is predicted to underlie the normal development of gut mucosa in the course of neoplastic progression (38). The continued presence of SMA in the pericrypt fibroblasts in hyperplastic polyps despite loss of HPP1 expression indicates that these cells are still present although altered.

In summary, demonstration of HPP1 methylation and loss of expression with RT-PCR in colorectal cancers is consistent with a role of hyperplastic polyps in the evolution of a subset of colorectal cancer (10, 16). The finding of methylation of HPP1 in most adenomas and cancers underlines a wider involvement of HPP1 in colorectal tumorigenesis. The structure and expression profile of HPP1 suggest that silencing by methylation may be implicated in an early disturbance of stromal–epithelial interaction.

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