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Villin Expression Is Frequently Lost in Poorly Differentiated Colon Cancer

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Abstract

Colorectal cancers (CRCs) are classified as having microsatellite instability (MSI) or chromosomal instability (CIN); herein termed microsatellite stable (MSS). MSI colon cancers frequently display a poorly differentiated histology for which the molecular basis is not well understood. Gene expression and immuno-histochemical profiling of MSS and MSI CRC cell lines and tumors revealed significant down-regulation of the intestinal-specific cytoskeletal protein villin in MSI colon cancer, with complete absence in 62% and 17% of MSI cell lines and tumors, respectively. Investigation of 577 CRCs linked loss of villin expression to poorly differentiated histology in MSI and MSS tumors. Furthermore, mislocalization of villin from the membrane was prognostic for poorer outcome in MSS patients. Loss of villin expression was not due to coding sequence mutations, epigenetic inactivation, or promoter mutation. Conversely, in transient transfection assays villin promoter activity reflected endogenous villin expression, suggesting transcriptional control. A screen of gut-specific transcription factors revealed a significant correlation between expression of villin and the homeobox transcription factor Cdx-1. Cdx-1 overexpression induced villin promoter activity, Cdx-1 knockdown down-regulated endogenous villin expression, and deletion of a key Cdx-binding site within the villin promoter attenuated promoter activity. Loss of Cdx-1 expression in CRC lines was associated with Cdx-1 promoter methylation. These findings demonstrate that loss of villin expression due to Cdx-1 loss is a feature of poorly differentiated CRCs.

Colorectal cancer (CRC) can be broadly classified as those displaying microsatellite instability (MSI) or chromosomal instability (CIN); herein referred to as microsatellite stable (MSS).¹ MSI colon cancers can be further separated into familial (Lynch syndrome) or sporadic MSI. Patients with Lynch syndrome have inherited mutations in one of six DNA mismatch repair genes, although mutations in MLH1 and MSH2 account for the majority (~90%) of cases.^{1,2} In Lynch syndrome, colonic tumors typically arise in the fourth decade of life, following loss of heterozygosity of the wild-type allele.¹ In comparison, sporadic MSI is driven largely by epigenetic silencing of the MLH1 locus with significantly later tumor onset.³ Inactivation of DNA mismatch repair genes results in acquisition of a mutator phenotype, which in turn drives tumorigenesis through mutation of key oncogenic and tumor suppressor signaling pathways including Wnt, Ras/BRAF, transforming growth factor- β (TGF- β), and PI3K.

Several cytogenetic and epigenetic differences exist between MSS/CIN and MSI tumors. In particular, MSS/CIN tumors display gains and losses of large chromosomal regions and multiple chromosomal rearrangements, whereas MSI tumors are largely diploid. Conversely, MSI tumors have a high mutation rate, and sporadic MSI tumors also display a high frequency of methylation-mediated tumor-suppressor gene inactivation.^{1,4,5} The molecular pathways that are genetically altered in MSS/CIN and MSI tumors are largely similar, although the specific mutations that deregulate these pathways do differ between these tumor subsets. For example, disruption of TGF- β signaling occurs primarily via mutations within SMAD4 in MSS/CIN tumors and via mutations in TGF- β -RII in MSI tumors.⁶ These differences are largely due to the more frequent occurrence of mutations in genes containing repetitive elements (microsatellites), such as TGF- β -RII⁷ and Bax,⁸ in MSI tumors.

Differences in tumor site, prognosis, response to systemic therapy and histopathological presentation have also been described between MSS/CIN and MSI tumors. MSI tumors occur more frequently in the right colon, and regardless of the depth of tumor invasion have a decreased likelihood of metastasizing to regional lymph nodes and distant organs.⁹ Differences in response to 5-fluorouracil-based adjuvant chemotherapy have also been described, with some, but not all studies suggesting MSI tumors derive less benefit from 5-fluorouracil-based therapy.^{10,11} Conversely, MSI tumors have been suggested to respond better to irinotecan.¹² Finally, MSI tumors frequently exhibit high lymphocytic infiltrates and poor differentiation grade compared to MSS/CIN tumors.^{2,13,14} The mechanistic basis for the loss of cellular differentiation in MSI colon cancers is poorly understood.

To identify novel regulators of MSS/CIN versus MSI colon cancer progression, with particular focus on the identification of factors that might mediate the poorly differentiated histology of MSI colon cancers, we searched for genes differentially expressed between these two colon cancer subclasses by gene expression profiling of a panel of human MSS/CIN and MSI colon cancer cell lines. Using this approach we identified the villin (*VILL*) gene as significantly down-regulated in expression in MSI tumors.

Villin is a 95 kDa protein that belongs to the Gelsolin family of calcium-regulated actin-binding proteins.^{15,16} Members of this family contain three or six homologous copies of a

conserved 120 amino acid gelsolin-like domain. Villin has six such domains, with approximately 45% sequence identity.¹⁶ Villin is a major component of the brush border cytoskeleton and functions in the capping, severing, and bundling of actin filaments.¹⁶ The expression pattern of villin is restricted largely to the intestinal epithelium and kidney proximal tubules and villin expression is used in the histopathological diagnosis of colon cancer.¹⁷ Villin derives its name from its localization to microvilli, the thin membrane extensions located on the apical membrane of polarized epithelial cells, which provide additional surface area for the absorption of nutrients. Microvilli are sustained by bundles of parallel actin filaments that are organized by multiple actin-binding proteins, including villin.

In this study we demonstrate that villin expression is frequently lost in MSI colon cancers and that this loss is linked to poorly differentiated histology in both MSI and MSS/CIN colon cancers. Furthermore, we demonstrate that villin loss is mediated at the transcriptional level due in part to loss of the key transcriptional regulator, Cdx-1. These findings indicate that loss of villin expression in tumors of unknown origin does not exclude the possibility of colon cancer.

Materials and Methods

Cell Lines and Cell Culture

The source of the human colon carcinoma cell lines used (Caco-2, Colo201, Colo205, Colo320, Dld-1, HCT116, HCT-15, HCT-8, HT29, LoVo, LS174T, RKO, SK-CO-1, SW1116, SW403, SW48, SW480, SW620, SW837, SW948, T84, WiDr, HT29-Cl.16E, HT29-Cl.19A, LIM1215, LIM2405, HCC2998, KM12, RW2982, RW7213, HuTu80, FET, ALA, GEO, LS1034, LS513, VACO5, Co115, HCA7, LS1080, and SNUC2B), and the methods of maintenance have been previously described.^{18–20} HCT116 WT and DNMT1 and 3b double knockout (DKO) cells were kindly provided by the Vogelstein Laboratory (Johns Hopkins University, Baltimore, MD) and have been previously described.²¹ The MSI status of the cell lines have been previously described.^{18,22}

Plasmids

The *VIL1* promoter reporter construct was generated by PCR amplification of a 1.7 kb fragment of the human villin promoter into the NotI and BamHI sites within the multiple cloning site of pGL3-Basic (Promega, Madison, WI). The 1.7 kb fragment comprised the region 1.2 kb upstream and 500 bp downstream of the villin transcription start site. Expression vectors for Cdx-1 and Cdx-2 were kindly provided by Dr. Philippe Soubeyran (INSERM, Marseille, France).²³

Microarray Analysis

Exponentially growing colon cancer cell lines were harvested and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). One to 5 μ g of total RNA was used for the probe (complementary RNA) preparation using the Affymetrix One Cycle protocol (Affymetrix, Santa Clara, CA), which was hybridized to human Affymetrix U133 Plus 2.0 arrays. Hybridization and image scanning were performed at the Peter MacCallum Cancer

Institute microarray facility. *CeI* files were normalized using GC-Robust Multiarray Average within Bioconductor.²⁴ Genes differentially expressed between MSS and MSI colon cancers were identified using an unpaired Student's *t*-test, with *P* < 0.05 considered statistically significant. The entire microarray database is available at Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>, accession number GSE35566).

Methylation Profiling of Colon Cancer Cell Lines

Methylation profiling of colon cancer cell lines was performed at the single nucleotide resolution using Human-Methylation27 beadchips from Illumina (San Diego, CA), which interrogates 27,578 CpG sites located within the proximal promoter regions of approximately 14,000 consensus coding sequence genes.

Quantitative Real-Time PCR

From each colon cancer cell line, 5 μ g of total RNA was converted into cDNA using anchored oligo dT primers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). From each cell line, 10 ng of cDNA was amplified with specific primers using the SYBR Green Core Reagents Kit and a 7900HT Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA). Abundance of gene expression was determined using the standard curve method and was expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Sequences of primers used are provided in Table 1.

Western Blot

Western blot analysis was performed as previously described.²⁵ Anti-villin monoclonal antibody was obtained from Novocastra (Newcastle Upon Tyne, UK) and used at a dilution of 1:2000 overnight.

Xenograft Experiments

For generation of colon cancer xenografts (5×10^6 colon cancer cells in a volume of 100 μ L of PBS) were mixed with an equal volume of matrigel, and injected subcutaneously into the right flank of severe combined immunodeficiency mice. Once palpable tumors were formed, animals were sacrificed, tumors were excised and fixed in formalin. Xenografts generated from multiple colon cancer cell lines were assembled on a tissue microarray for subsequent immunohistochemical analysis.

Tissue Microarrays and Immunohistochemistry

The generation of the tissue microarray comprising 577 human Dukes B and C colon cancers has been previously described.^{26–28} MSI status was determined by the absence of MLH1 or MSH2 staining by immunohistochemistry and determination of the length of the BAT-26 mononucleotide repeat as previously described.²⁶ Tissue microarrays were stained with an anti-villin Ab obtained from Novocastra (Newcastle Upon Tyne, UK) at a dilution of 1:50. Each sample was scored for villin staining intensity as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). For each sample, the percentage of positive cells was also scored, and the subcellular localization of villin (membrane, membrane and cytoplasmic, cytoplasmic, or nuclear) recorded.

Transient Transfection Assays and Small-Interfering RNA Knockdown Experiments

Colon cancer cells were transiently transfected with the villin promoter luciferase reporter construct using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). TK-Renilla was included to control for transfection efficiency. Luciferase activity was determined 24 hours post-transfection using the dual-luciferase reporter assay kit from Promega (Madison, WI). For small-interfering RNA experiments, cells were transfected using Lipofectamine 2000 and a pool of 4 Cdx-1 or Cdx-2 targeting small-interfering RNAs obtained from Dharmacon (Lafayette, CO). Cells were harvested at 24, 48, or 72 hours post-transfection.

Direct Sequencing

Genomic DNA from colon cancer cell lines was extracted using the Qiagen DNA extraction kit (Qiagen, Valencia, CA). Direct sequencing of the villin gene and promoter was performed by PCR amplification of all 20 coding exons in the villin gene, purification of the PCR product (ExoSap IT, Affymetrix), and sequencing by standard Sanger sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). Primer sequences are provided in Table 1.

Results

Villin Expression is Frequently Lost in MSI Colon Cancer Cell Lines and Primary Tumors

To identify novel genes differentially expressed between MSS and MSI colon cancers, the basal gene expression profile of 5 MSS and 5 MSI colon cancer cell lines were profiled using Affymetrix U133 Plus 2.0 microarrays. The entire microarray database is available at GEO (<http://www.ncbi.nlm.nih.gov/geo>, accession number GSE35566). Among the most highly differentially expressed genes was *VILL1*, which was reduced approximately ninefold in MSI cell lines (signal intensity, 3357 ± 2392 , and 376 ± 111 mean $\pm$ SD for MSS and MSI lines respectively; $P = 0.02$) (Figure 1A).

To validate this finding, villin mRNA expression was examined by quantitative real-time PCR in a larger panel of 40 colon cancer cell lines. Consistent with the microarray data, villin expression was 10-fold lower in MSI compared to MSS cell lines in this expanded panel ($P = 0.005$), and notably was undetectable by quantitative PCR in 10/16 MSI cell lines (Figure 1B). All MSS cell lines, with the exception of HuTu-80 demonstrated villin expression, although to varying degrees (Fisher's exact-test, $P = 0.01$) (Figure 1B). We also examined villin protein expression by Western blot in five villin-positive MSS/CIN lines, and five MSI cell lines that showed undetectable villin expression at the mRNA level. MSI cell lines lacking villin mRNA expression also demonstrated complete absence of villin protein expression (Figure 1C). To further confirm this finding these 10 cell lines were grown as xenografts in severe combined immunodeficiency mice and villin expression examined by IHC. Consistent with the mRNA and Western blot data, strong villin expression was observed in the five MSS/CIN lines, with minimal to no villin expression evident in the MSI lines (Figure 1D).

To confirm that villin expression was similarly down-regulated in primary MSI colon cancers, we examined villin mRNA expression in a cohort of 97 colon cancers of known MSI status that had previously been profiled using Affymetrix gene expression microarray analysis.²⁹ Consistent with the cell line findings, a significant reduction in *VILL1* expression in the MSI tumors (566 ± 370 ; $n = 36$) relative to both normal mucosa (1025 ± 723 ; $n = 16$) and MSS tumors (964 ± 346 ; $n = 61$; mean $\pm$ SD; $P < 0.001$, Student's *t*-test) was observed (Figure 2A). Villin expression was significantly down-regulated in both sporadically arising MSI tumors, as well as in early onset MSI tumors derived from Lynch syndrome patients (Figure 2A). To confirm this finding at the protein level, and to determine whether loss of villin expression was linked to clinicopathological features of colon cancers, we examined villin expression in a large tissue microarray, comprising 519 MSS and 58 MSI Dukes B and C colon cancers, generated at the University of Western Australia. Consistent with loss of villin mRNA expression, villin protein expression was significantly lower in MSI (1.4 ± 0.8) compared to MSS colon cancers (1.7 ± 0.7 , mean $\pm$ SD; $P < 0.001$ Student's *t*-test) (Figure 2B). Likewise, the percentage of villin-positive cells per tumor was significantly lower in MSI (48.1 ± 26.3) compared to MSS (60.7 ± 24.2 , mean $\pm$ SD; $P < 0.002$ Student's *t*-test) colon cancers (Figure 2C). Finally, the percentage of tumors showing minimal villin staining (staining intensity = <0.5) was significantly higher in the MSI subset (17.2%) compared to the MSS subset (5.4%) (Figure 2D). This finding was independently validated in a separate tissue microarray comprising 64 Dukes C MSS and 10 Dukes C MSI tumors generated in Finland, where the villin staining intensity was 1.5 ± 1.0 in MSS tumors compared to 0.5 ± 0.7 in MSI tumors (mean $\pm$ SD; $P = 0.002$, Student's *t*-test) (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Representative tumors displaying villin staining intensities 0–3 are also shown (Figure 2E).

Villin Expression Is Preferentially Down-Regulated in Poorly Differentiated Colon Cancers

Villin is a cytoskeletal protein that is localized to microvilli located on the apical surface of differentiated intestinal epithelial cells.¹⁵ We examined, therefore, whether loss of villin expression was linked to tumor differentiation grade, using the World Health Organization grading scheme that is based on the proportion of glandular differentiation within the tumor. As poorly differentiated histology is an established feature of MSI colon cancers, we performed separate analyses on the MSI and MSS tumors. Villin staining intensity was significantly lower in tumors with poorly differentiated histology (minimal to no glandular differentiation) compared to those with well/moderately differentiated histology (presence of glandular differentiation) in both the MSI (1.1 ± 0.8 versus 1.6 ± 0.8 , respectively, mean SD; $P = 0.045$ Student's *t*-test) and MSS (1.6 ± 0.8 versus 1.8 ± 0.7 , respectively; $P = 0.003$) subsets (Figure 3, A and B). Likewise, the percentage of tumors with minimal to no villin staining (staining intensity of 0.5 or less) was higher in tumors with poorly differentiated compared to well/moderately differentiated histology for both the MSI (27.3 versus 11.1%; $P = 0.114$, χ^2 test) and MSS (4.3 versus 9.6%; $P = 0.049$, χ^2 test) subsets, although this was not statistically significant in the MSI group, possibly due to the small number of tumors in this category (Figure 3, A and B). Villin staining of representative well/moderately differentiated and poorly differentiated colon cancers are also shown (Figure 2, C and D, respectively).

Loss of Villin Membrane Staining Is Associated with Worse Outcome in Colon Cancer

Next we examined whether villin expression was linked to overall patient survival. As MSI status alone is known to be linked to improved patient survival, once again we analyzed the MSI and MSS subgroups separately. No association between villin staining intensity and overall survival was observed in either the MSS or MSI groups (Figure 4, A and B, respectively).

We also observed that the localization of villin expression, which in the normal colonic epithelium is primarily localized to the cell membrane was frequently mislocalized in colon tumors (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). To determine whether mislocalization of villin away from the cell membrane was a prognostic factor, tumors were also classified as having exclusively membrane staining or those with mislocalized villin staining. Separation of tumors into these categories demonstrated that in the MSS subgroup, tumors with exclusively membrane staining had a significantly better outcome (Figure 4C). In the MSI subset, tumors that had exclusively membranous villin staining displayed a tendency for improved survival; however, this was not statistically significant ($P = 0.211$) (Figure 4D). This difference was maintained when only the moderately differentiated MSS tumors were analyzed, although this narrowly missed statistical significance ($P = 0.058$) (Figure 4E), but was not significantly different when the poorly differentiated MSS subset was independently analyzed (Figure 4F).

Loss of Villin Expression in MSI Tumors Is Not Due to Coding Sequence or Promoter Mutation

Next we sought to determine the mechanism by which villin expression is lost in MSI colon cancers. Multiple mechanisms can be envisioned, including truncating mutations within the coding sequence, mutations within the promoter, epigenetic inactivation of the villin promoter, or absence/presence of a key transcriptional regulator. We, therefore, systematically addressed each of these possibilities.

First, to determine whether loss of villin expression is mediated by truncating mutation, the full coding sequence of the villin gene (exons 2–20) were directly sequenced in 3 villin-negative MSI cell lines (HCT116, LIM2405, and Dld). However, no mutations were detected in any of the lines, indicating that truncating mutations is not the basis for villin loss (data not shown).

Second, we determined whether mutations within the villin promoter could account for the loss of villin expression in MSI cell lines. Given the susceptibility of repeat regions to mutation in MSI deficient tumors, we focused on two repeat elements within close proximity to the transcription start site of the villin gene. Direct sequencing of a short 7G repeat located 35 bp from the transcription start site in 5 villin-negative and 5 villin-positive MSI cell lines failed to identify any mutations in any of these cell lines. Sequencing of a longer A14 repeat located 185 bp upstream of the transcription start site identified mono-, di- or trinucleotide deletions of this repeat in 6 of the 10 MSI lines. These mutations, however, did not correlate with villin expression, with 2 of 5 villin promoter WT lines not expressing endogenous villin, and 3 of 5 villin promoter-mutant lines strongly expressing endogenous

villin (Table 2) (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). No mutations in either repeat element were identified in any of the five villin-positive MSS cell lines examined (Table 2; see also Supplemental Figure S3 at <http://ajp.amjpathol.org>).

To further confirm that mutations in the A14 repeat do not impact villin promoter activity, we cloned a 1.7 kb fragment of the villin promoter encompassing a region 1.5 kb upstream through 200 bp downstream of the transcription start site, and containing the A14 repeat, into the pGL3 basic luciferase reporter vector. We also generated a construct that contained the same 1.7 kb region, but harboring a tri-adenosine deletion mutation (A11) of the A14 repeat element, similar to that observed in LIM2405 cells. Transfection of each of these constructs into three villin-positive colon cancer cell lines failed to demonstrate any difference in promoter reporter activity, indicating deletion mutations within this repetitive element has minimal impact on villin expression (see Supplemental Figure S4 at <http://ajp.amjpathol.org>).

Loss of Villin Expression Is Not Mediated by Epigenetic Inactivation

Next, to determine whether loss of villin expression is mediated by promoter methylation, villin-negative HCT116 cells were treated with the DNA methyltransferase inhibitor (5-azacytidine) for 72 or 120 hours. However, no induction of villin mRNA or protein was observed (see Supplemental Figure S5, A and C at <http://ajp.amjpathol.org>). In contrast, azacytidine treatment induced expression of secreted frizzled-related protein 1 (SRFP1) (see Supplemental Figure S5B at <http://ajp.amjpathol.org>), which is known to be epigenetically inactivated in HCT116 cells.³⁰ To confirm this finding, we also examined villin expression in a clonal derivative of HCT116 cells, lacking the DNMT1 and DNMT3b genes.²¹ These cells display global DNA hypomethylation and re-express a number of epigenetically inactivated genes,²¹ including SRFP1 (see Supplemental Figure S5E at <http://ajp.amjpathol.org>). However, as shown (see Supplemental Figure S5D at <http://ajp.amjpathol.org>), the expression of villin was not detected in either parental or HCT116-DNMT1/DNMT3b double knockout cells. Finally, examination of the villin promoter failed to identify the presence of a CpG island, collectively indicating promoter methylation is unlikely to be a major mechanism of villin inactivation in MSI colon cancer cell lines.

Loss of Endogenous Villin Expression Is Linked to Reduced Promoter Reporter Activity

Next we addressed the possibility that loss of villin expression in MSI tumors may be the consequence of a loss of a key transcriptional regulator. We rationalized that if the loss of villin expression is due to the loss of a key regulatory factor, activity of a transiently transfected villin promoter-reporter construct should reflect endogenous villin expression. To address this, the 1.7 kb villin promoter reporter construct was transiently transfected into 5 villin-positive and 5 villin-negative colon cancer cell lines, as well as four noncolon lines that do not express villin. Villin promoter reporter activity closely paralleled endogenous villin expression (Figure 5B; $r = 0.83$, Pearson's correlation coefficient; $P < 0.005$), strongly suggesting that loss of villin expression in MSI colon cancers is due to the absence of a key transcriptional regulator. In comparison, no significant difference in luciferase activity driven by a Sp1/Sp3 reporter construct was evident between the three groups of cell lines (Figure 5C).

Loss of Villin Expression Correlates with Loss of Cdx-1 Expression in MSI Cell Lines

Next we sought to identify putative transcription factors whose loss of expression in MSI colon cancer may mediate loss of villin expression. From a survey of the literature, we identified 10 transcription factors previously shown to play a role in regulating intestinal cell differentiation (*TCF7L2*, *IHH*, *HNF1a*, *HNF4a*, *Cdx-1*, *Cdx-2*, *KLF4*, *ELF3*, *ISX*, and *GATA6*).³¹ Primers to each of these transcription factors were designed and their expression levels correlated with that of villin across a panel of 40 colon cancer cell lines. Four transcription factors, (*ELF3*, *ISX3*, *GATA6*, and *Cdx-1*) whose expression was positively and significantly correlated with villin expression, were identified, of which the correlation was most significant for the homeobox transcription factor, *Cdx-1* ($r = 0.58$; $P < 0.001$) (Figure 6).

Cdx-1 Regulates Villin Expression in Colon Cancer Cells

To determine whether exogenous expression of *Cdx-1* can directly induce villin expression, we co-expressed *Cdx-1* with the villin promoter reporter construct, in *VIL1*-negative RKO cells. *Cdx-1* overexpression strongly induced villin promoter activity in RKO cells indicating *Cdx-1* regulates villin expression (Figure 7A). To determine whether *Cdx-1* can regulate endogenous villin expression, we examined the effect of Cdx-1 knockdown on villin mRNA expression in villin-positive SW948 colon cancer cells. Transient transfection of SW948 cells with Cdx-1-targeting small-interfering RNAs resulted in a 77% down-regulation of Cdx-1 levels (Figure 7B). In parallel, expression of endogenous villin mRNA expression was decreased by 52% (Figure 7C).

Examination of the 1.7 kb villin promoter fragment identified the presence of 10 putative *Cdx-1* binding sites. To determine whether *Cdx-1* directly induces villin expression, we generated a promoter fragment lacking 9 of the 10 putative *Cdx-1* binding sites (Figure 7D). Notably, transfection of this truncated villin promoter construct into SW948 cells demonstrated similar levels of expression compared to the 1.7kb villin construct, indicating the majority of these putative Cdx binding sites are nonfunctional. Conversely, site-directed mutagenesis of the remaining Cdx binding site, located 450 bp downstream of the transcription start site, resulted in a 52% decrease in villin promoter activity, indicating this site is required for maximal villin expression. To further investigate the importance of this site in Cdx-1 induction of villin promoter activity, we co-transfected RKO cells with these villin promoter reporter constructs along with Cdx-1. Cdx-1 strongly induced activity of the wild-type villin promoter reporter, although this effect was markedly attenuated on deletion of the -450 Cdx binding site (Figure 7E). Collectively, these findings demonstrate that Cdx-1 directly regulates villin expression in colon cancer cells.

Loss of Cdx-1 Expression Is Linked to Promoter Methylation

Cdx-1 expression has been shown to be down-regulated in colon cancer and this has been linked to hypermethylation of the Cdx-1 promoter.^{32–34} We have previously performed microarray-based whole genome methylation profiling of 27 colon cancer cell lines, providing an opportunity to determine whether down-regulation of Cdx-1 expression is linked to promoter methylation in our cell line panel. Correlation of Cdx-1 promoter methylation status with expression across the cell line panel demonstrated a significant

inverse correlation ($R = -0.56$; $P = 0.0024$) (Figure 7F), indicating loss of Cdx-1 expression is associated with promoter methylation.

Discussion

In this study, we demonstrate that the expression of the actin-binding cytoskeletal protein villin is frequently lost in MSI colon cancer cell lines, as well as primary MSI tumors. MSS/CIN colon cancers also demonstrated a broad range of villin staining intensity and subcellular localization. In both MSI and MSS colon cancers, weak villin expression was significantly associated with poorly differentiated histology. Notably, ~27% of MSI and ~9% of MSS colon cancers with poorly differentiated histology showed minimal to no villin expression. This finding is of clinical significance, because immunohistochemistry for villin expression is used to indicate colonic origin in the diagnosis of metastatic carcinomas of unknown origin.^{17,35–37} These findings indicate that absence of villin expression in a poorly differentiated tumor does not exclude colorectal cancer.

Whether the poorly differentiated histology (lack of glandular structure) of tumors lacking villin expression is mediated by villin loss remains to be determined. At the cellular level, villin has been shown to be important for the maturation phenotype of Caco-2 colon cancer cells *in vitro*, as villin knockdown resulted in disrupted microvillus structure and brush border assembly.³⁸ In contrast, no obvious phenotype of the normal colonic epithelium, including brush border development was observed in villin knockout mice,³⁹ and it has been suggested that functional redundancy between the actin bundling proteins, plastin-1, and espin may compensate for villin loss in the normal intestine.⁴⁰ Nevertheless, these *in vivo* findings suggest that villin loss alone may not be sufficient to induce loss of glandular differentiation in colon cancer. Instead, given our finding that villin down-regulation in colon cancer is a consequence of loss of Cdx-1 expression, a likely possibility is that multiple proteins that are Cdx-1 targets will be down-regulated in parallel, and that collectively this results in a loss of glandular differentiation. Consistent with this possibility, the intestinal-specific structural protein (keratin-20) is also lost in a number of cell lines that lack Cdx-1 and villin expression,⁴¹ and has been shown to be a direct target of Cdx-1.⁴¹

Interestingly, while villin plays a role in the actin bundling required for microvillus structure, a number of studies have demonstrated that villin can also induce actin severing at physiologically relevant calcium concentrations.^{42,43} Notably, this function of villin has been linked to the promotion of cell migration *in vitro*, indicated by the significantly diminished rates of cell scattering and wound repair in response to hepatocyte growth factor treatment,⁴⁴ and the enhanced rate of cell migration after overexpression of villin in Hela cells.⁴⁵ Furthermore, Ferrary et al³⁹ observed a markedly increased sensitivity of villin^{-/-} mice to dextran sodium sulfate-induced colonic injury, suggestive of reduced wound healing capacity and impaired cell migration rates in villin-deficient mice. Given its role in cell migration, one possibility was that villin may play a pro-metastatic role in colorectal tumorigenesis. However, no association between loss of villin expression and poorer outcome was observed in this study. On the other hand, tumors that displayed mislocalized villin expression, away from its normal localization to the cell membrane, were associated with poorer outcome. An intriguing possibility worthy of further investigation, therefore, is

whether villin may differentially induce actin bundling versus severing, depending on the subcellular compartment to which it is localized.

This study also examined whether the molecular basis for loss of villin expression was genetic, epigenetic, or transcriptional. First, we eliminated a genetic basis for villin loss after the failure to identify any mutations in the coding sequence of the villin gene. We also examined the possibility that promoter mutations, particularly in two mononucleotide repeats in the proximal villin promoter might explain villin loss in MSI cell lines. Although mutations in the long mononucleotide repeat (A14) within the villin promoter were observed in 60% on MSI colon cancer cell lines, these mutations did not correlate with villin expression, indicating they are not the basis for villin loss.

An epigenetic basis for villin loss was eliminated based on three pieces of evidence. First, the villin promoter lacks a CpG island, suggesting it is unlikely to be epigenetically regulated. Second, treatment of villin-negative colon cancer cell lines with the DNA methyltransferase inhibitor (5-azacytidine) failed to induce villin expression. Finally, HCT116 DNMT1/3b knockout cells, where targeted inactivation of the DNMT1 and DNMT3b genes induces global DNA hypomethylation and re-induction of multiple epigenetically silenced genes,²¹ failed to demonstrate reactivation of villin expression.

On the other hand, we demonstrated that the mechanistic basis for loss of villin expression is at least partially due to transcriptional deregulation. This was suggested by the finding that activity of a transiently transfected villin promoter reporter construct closely reflected endogenous villin expression across a panel of colon cancer and noncolon cancer cell lines. Screening of a number of transcription factors implicated in the regulation of intestinal cell differentiation identified the homeobox transcription factor Cdx-1 as the most strongly correlated with villin expression. Furthermore, transient overexpression of Cdx-1 into Cdx-1 and villin-negative cells induced villin promoter activity, whereas knockdown of Cdx-1 decreased endogenous villin expression. Finally, deletion of a Cdx binding site within the first intron of villin reduced villin promoter activity by >50%, collectively suggesting Cdx-1 directly regulates villin expression. Importantly, loss of expression of Cdx-1 has previously been linked to promoter methylation in primary colon cancers and colon cancer cell lines.^{32–34} Notably, all of the colon cancer cell lines previously shown to lack Cdx-1 expression due to promoter methylation, including HCT116, RKO, and SW48,^{34,41} were found to lack villin expression in our study. Consistent with these findings, we demonstrated a significant inverse correlation between Cdx-1 promoter methylation and villin expression.

We demonstrated a direct role for Cdx-1 in regulating villin expression, although it is notable that despite elimination of all putative Cdx-1 binding sites, some villin promoter activity was still retained, indicating additional transcription factors may also contribute to villin expression. Interestingly, Yamamichi et al⁴⁶ reported a correlation between expression of the highly related Cdx-2 transcription factor and villin expression in a cohort of gastric tumors. Furthermore, they demonstrated that Cdx-2 over-expression enhanced villin expression, whereas Cdx-2 knockdown inhibited villin expression in SW480 colon cancer cells.⁴⁶ Notably, intestinal-specific conditional knockout studies have demonstrated redundant functions between Cdx-1 and Cdx-2, particularly in relation to features of

intestinal differentiation,^{47,48} suggesting these factors may both play a role in villin regulation. In addition to Cdx-1, we identified three other transcription factors (*ELF3*, *ISX3* and *GATA6*), whose expression levels correlated significantly with that of villin. Whether these factors directly regulate villin or whether they do so by regulating or interacting with Cdx-1 needs to be individually determined.

In summary, this study demonstrates that the expression of the intestinal-specific differentiation marker villin is frequently lost in colon cancer, particularly MSI colon cancers, and most frequently in colon cancer with poorly differentiated histology. Furthermore, deregulated subcellular localization of villin, away from its normal location to the cell membrane, was associated with poorer patient outcome. Finally, we demonstrate that loss of villin expression is mediated at the transcriptional level, due in part to loss of the key intestinal cell differentiation regulator, Cdx-1. These findings are of clinical significance as they demonstrate that loss of villin expression cannot be used to eliminate colon cancer in the diagnosis of tumors of unknown origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Villin expression is significantly down-regulated in microsatellite instability (MSI) colon cancer cell lines. **A:** Villin mRNA expression in five microsatellite stable (MSS) and 5 MSI colon cancer cell lines profiled using Affymetrix microarrays. **B:** Villin mRNA expression in a panel of 40 colon cancer cell lines (24 MSS and 16 MSI) assessed by quantitative real-time PCR. **C, D:** Validation of loss of villin protein expression in MSI cell lines by Western blot analysis (**C**) and immunohistochemistry (**D**). For IHC studies, five MSS and five MSI cell lines were grown as xenografts in severe combined immunodeficiency mice, and the tumors were excised and the formalin was fixed. Villin expression was determined by IHC using a commercially available anti-villin antibody.

Figure 2.

A: Villin mRNA expression in primary microsatellite instability (MSI) and microsatellite stable (MSS) colon cancers. Gene expression differences between normal colonic mucosa, MSS, and MSI colon tumors were compared using Affymetrix microarrays. Villin expression was significantly down-regulated in both sporadically arising and early onset MSI tumors derived from Lynch syndrome patients. **B–E:** Loss of villin protein expression in MSI colon cancer. Tissue microarrays comprising 519 MSS and 58 MSI tumors were stained for villin expression using an anti-villin antibody. Villin expression was assessed in each sample by scoring of the (**B**) overall staining intensity and (**C**) the percentage of villin-positive cells. **D:** The percentage of MSS and MSI tumors with an overall villin staining intensity of 0.5 or less. **E:** Representative tumors displaying various villin staining intensities of 0 to 3, as described in Materials and Methods. * $P < 0.001$, t -test; $^{\dagger}P = 0.0002$, t -test; $^{\ddagger}P < 0.00001$, t -test; $^{\S}P = 0.0006$, χ^2 .

Figure 3.

Loss of villin expression is most frequently observed in poorly differentiated colon cancers. Villin staining intensity and the percentage of colon cancers with villin staining intensity of 0.5 in well/moderately differentiated and poorly differentiated microsatellite instability (MSI) (**A**) and microsatellite stable (MSS) (**B**) colon cancers. **C, D:** Villin expression in representative well/moderately differentiated (**C**) and poorly differentiated (**D**) colon cancers. * $P < 0.05$; $^{\dagger}P < 0.005$, Student's t -test; and $^{\S}P < 0.05$, χ^2 test.

Figure 4.

Prognostic significance of villin expression and subcellular localization in Dukes B/C colon cancer. Kaplan-Meier analysis demonstrating villin staining intensity is not prognostic in microsatellite stable (MSS) (A) or microsatellite instability (MSI) (B) colon cancer. Prognostic significance of localization of villin exclusively to the cell membrane in MSS (C) and MSI (D) colon cancer. Prognostic significance of localization of villin exclusively to the cell membrane in moderately differentiated (E) and poorly differentiated (F) MSS tumors.

Figure 5.

Villin promoter luciferase reporter activity reflects endogenous villin expression. **A:** Endogenous villin mRNA expression in four noncolon cancer cell lines, and five villin-negative and five villin-positive colon cancer cell lines. **B:** Villin promoter reporter activity in the same cell line panel. Each cell line was transfected with the villin promoter reporter along with TK-Renilla as a control for transfection efficiency. **C:** Sp-1 reporter activity in the cell line panel. Each cell line was transfected with the villin promoter luciferase reporter along with TK-Renilla as a control for transfection efficiency. Values shown in **B** and **C** are mean $\pm$ SEM of a representative experiment performed in triplicate.

Figure 6.

Correlation of endogenous mRNA expression of 10 transcription factors (*TCF7L2*, *IHH*, *HNF1a*, *HNF4a*, *Cdx-1*, *Cdx-2*, *KLF4*, *ELF3*, *ISX*, and *GATA6*) implicated in the regulation of intestinal cell differentiation, and villin, in a panel of 40 colon cancer cell lines. Correlation was assessed by computing the Spearman's correlation coefficient.

Figure 7.

Cdx-1 regulates villin expression. **A:** Transient Cdx-1 overexpression induces villin promoter activity in RKO cells. Cells were transfected with empty vector control or a Cdx-1 expression vector in combination with the villin promoter luciferase construct, and villin reporter activity determined using the dual luciferase reporter assay. **B, C:** Cdx-1 knockdown represses endogenous villin expression. SW948 cells were transiently transfected with Cdx-1 targeting small-interfering RNA (siRNA) or nontargeting control siRNAs for 48 hours, and the effect on **(B)** Cdx-1 and **(C)** villin mRNA expression was determined by quantitative PCR. Values shown are mean $\pm$ SEM of a representative experiment performed in triplicate. **D:** Deletion of all Cdx-1 binding sites in the villin promoter construct attenuates villin promoter reporter activity. The villin promoter luciferase reporter comprising 1200 bp upstream and 500 bp downstream of the villin transcription start site (TSS) (villin 1702), or the various deletion constructs shown, were transiently transfected into SW948 cells and villin promoter activity assessed using the dual luciferase reporter assay. **E:** RKO cells were transiently transfected with a series of villin promoter reporter constructs and Cdx-1, and villin promoter activity assessed using the dual luciferase reporter assay. Values shown are mean $\pm$ SD of a representative experiment performed in triplicate. **F:** Cdx-1 promoter methylation status inversely correlates with Cdx-1 expression across a panel of 27 colon cancer cell lines ($r = -0.56$; $P = 0.0024$). EV, empty vector; NT, non-targeting.

Table 1**Villin Sequencing and Real-Time PCR Primers Used in the Study**

Exon	Forward primer	Reverse primer
Villin sequencing primers		
1	Noncoding	Noncoding
2	5'-TGCTAGACACTGCGGATTG-3'	5'-GAAAGGGAAAGGGAGACCAG-3'
3	5'-TCACTCATCCCTTCCTTCC-3'	5'-ATTGTGTCTTGGGGTGAAGC-3'
4	5'-GAGGTCACACAGAGCTGGAG-3'	5'-CCTGCTCCAAGTGTCTTCC-3'
5	5'-TCATTGACCTCGCCTACTCC-3'	5'-AGACCCCACTCAGAGCTCAC-3'
6	5'-GAACGTGGTAGCTGGAGAGG-3'	5'-ACACACACGCAAACCTCAAC-3'
7	5'-GTGTAGGGTGGCAGACACTG-3'	5'-CACTGCAGGACAGAAGCAAG-3'
8	5'-TCAAGGTGCACTCAAACTG-3'	5'-ACTGGGAAAGCCTCACACAC-3'
9	5'-CACGAGGTAAGAGGGTCTGG-3'	5'-GCACTATGCCCTGGTCTGAG-3'
10	5'-GGGTTAGGTTGGGGATTAGC-3'	5'-CCAGAGCCAGCGTAAAGTC-3'
11	5'-GTTGTGAGGACCTGGGAGAC-3'	5'-ACCTGACCTGCCAAACGTAG-3'
12	5'-AGTGGGGAAGTGCAGGTATG-3'	5'-CCTCATAGCCAAAGCAGTCC-3'
13	5'-TGAGGATGGAGTTGTGCTG-3'	5'-TGCCAGACACCTCAGTTCAG-3'
14	5'-GAGCATCAGAGGAGCTCAGG-3'	5'-ACTTAGTTGGCCAGGGGTTTC-3'
15	5'-TGAACCTGGGCAGTGAATG-3'	5'-TCAGTCCATGCCTGCTGTAG-3'
16	5'-GCATTTTGCTGGAGATGACC-3'	5'-CTCCCCACCTTCCTTCTTTC-3'
17	5'-AGGGTCAAGGTCAGGGTTTC-3'	5'-TGGCCTTAGTGCAGCCTTAC-3'
18	5'-CAGCCTCAGCCTTCATATCC-3'	5'-TCTTCTCTGCAGCGTCTTG-3'
19	5'-GAGGCTTCTTGAAGGTGGTG-3'	5'-TTCATCCCCAGCACCTAGTC-3'
20	5'-CTGAGGGTGAGGGAGGAAG-3'	5'-TGCAATACCTCAGGATAGAAAC-3'
Primers used in quantitative real-time PCR		
Beta-actin	5'-CACCTTCACCGTTCCAGTTT-3'	5'-GATGAGATTGGCATGGCTTT-3'
Villin	5'-AGCCAGATCACTGCTGAGGT-3'	5'-TGGACAGGTGTTCTCCTTC-3'
GAPDH	5'-ATGGAAATCCCATCACCATCTT-3'	5'-CGCCCCACTTGATTTTGG-3'
Cdx1	5'-ACTGAACGGCAGGTGAAGA-3'	5'-ACAGGCATTGGAGAGGAGGT-3'
Cdx2	5'-AGAGGGACTCAAGGGAAGG-3'	5'-GGTCTGGGAAGGGAAGAGAA-3'
HNF1A	5'-GTGTGGCGAAGATGGTCAAGT-3'	5'-CTGTGGGATGTTGTGCTGCT-3'
ELF3	5'-AGCGATGGTTTTCTGACTG-3'	5'-GCGTGCTTGCTCTTCTTG-3'
TCF7L2	5'-CACACTTACCAGCCGACGTA-3'	5'-TCCTGTCTGATTGGGTACA-3'
Isx	5'-CAGGAAGGAAGGAAGAGCAA-3'	5'-TGGGTAGTGGGTAAAGTGGAA-3'
IHH	5'-AAGGACGAGGAGAACACAGG-3'	5'-ACCGAGATAGCCAGCGAGTT-3'
GATA6	5'-CAAACCAGGAAACGAAACC-3'	5'-AAGAGGTGGAAGTTGGAGTCA-3'
HNF4a	5'-CATCAGAAGGCACCAACCTC-3'	5'-GTCTTTGTCCACCACGCACT-3'
KLF4	5'-CAAGCCAAAGAGGGGAAGAC-3'	5'-GTGTGCCTTGAGATGGGAAC-3'

Table 2

A14 Repeat Element Status and Endogenous Villin mRNA Expression in MSI and MSS Colon Cancer Cell Lines

Cell line	G7 repeat	A14 repeat	MSS/MSI	Villin mRNA
DLD-1	WT	WT	MSI	—
HCT116	WT	MUT	MSI	—
LIM2405	WT	MUT	MSI	—
RKO	WT	MUT	MSI	—
SW48	WT	WT	MSI	—
HCT-15	WT	WT	MSI	+
HCT-8	WT	WT	MSI	+
KM12	WT	MUT	MSI	+
LIM1215	WT	MUT	MSI	+
LS174T	WT	MUT	MSI	+
Caco-2	WT	WT	MSS	+
HT29	WT	WT	MSS	+
HCC2998	WT	WT	MSS	+
SK-CO-1	WT	WT	MSS	+
T84	WT	WT	MSS	+

An A14 repeat element in the *VILL1* promoter is frequently mutated in MSI colon cancer cell lines, but the mutation is not linked to villin mRNA expression.

MSI, microsatellite instability; MSS, microsatellite stable; MUT, mutant; WT, wild-type.