The Paracrine Hormone for the GUCY2C Tumor Suppressor, Guanylin, Is Universally Lost in Colorectal Cancer

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Abstract

Background: Although colorectal cancer is a disease characterized by sequential accumulation of mutations in epithelial cells, mechanisms leading to genomic vulnerability contributing to tumor initiation remain undefined. GUCY2C has emerged as an intestine-specific tumor suppressor controlling epithelial homeostasis through circuits canonically disrupted in cancer. Surprisingly, the GUCY2C tumor suppressor is universally overexpressed by human colorectal cancer cells. This apparent paradox likely reflects silencing of GUCY2C through loss of its paracrine hormone guanylin. Here, we quantified expression of guanylin mRNA and protein in tumors and normal epithelia from patients with colorectal cancer.

Methods: Guanylin mRNA was quantified in tumors and normal adjacent epithelia from 281 patients by the reverse transcriptase-polymerase chain reaction. Separately, the guanylin protein was quantified by immunohistochemistry in 54 colorectal tumors and 30 specimens of normal intestinal epithelium.

Results: Guanylin mRNA in colorectum varied more than a 100-fold across the population. Guanylin mRNA was reduced 100- to 1,000-fold in >85% of tumors compared with matched normal adjacent mucosa (P < 0.001). Loss of guanylin mRNA was greatest in tumors from patients <50 years old (P < 0.005) and with the highest expression in normal adjacent mucosa (Spearman correlation coefficient = 0.61; P < 0.001). In a separate validation cohort, guanylin protein was detected in all 30 normal colorectal mucosa specimens, but in none of 54 colorectal tumors.

Conclusions: Colorectal cancer may initiate as a disease of paracrine hormone insufficiency through loss of guanylin expression, silencing the GUCY2C tumor suppressor and disrupting homeostatic mechanisms regulating colorectal epithelia cells.

Impact: Intestinal tumorigenesis may be prevented by oral GUCY2C hormone replacement therapy. Cancer Epidemiol Biomarkers Prev; 23(11); 2328–37. ©2014 AACR.

Introduction

Although sporadic colorectal cancer is the fourth leading cause of cancer and the second leading cause of cancer-related death worldwide (1), mechanisms underlying tumor initiation remain incompletely defined. The oncogenic view suggests that colorectal cancer is a disease of sequential accumulation of mutations in a defined set of genes in the single layer of epithelial cells lining the colon and rectum (2). In turn, these mutations contribute to the spatiotemporal continuum of transformation, from loss of normal growth control mediating the formation of benign adenoma, to the ability of tumor cells to invade and metastasize (3). Although these steps in tumor progression have been characterized, initiating mechanisms at the center of colorectal cancer pathogenesis that establish the susceptibility of intestinal epithelial cells to undergo sequential mutagenesis remain unknown.

Guanylyl cyclase C (GUCY2C) is a member of the membrane-bound guanylyl cyclase family of transmembrane receptor enzymes that is selectively expressed in brush border membranes of intestinal epithelial cells from the gastroduodenal junction to the end of the rectum (4). The cognate ligands for this receptor include the endogenous paracrine hormones uroguanylin and guanylin, expressed in small and large intestine, respectively, and the exogenous heat-stable enterotoxin (ST) produced by diarrheagenic enterotoxigenic bacteria (5). Interaction of
these ligands with the extracellular receptor binding domain is required to activate the cytoplasmic catalytic domain that synthesizes cyclic GMP (cGMP) from GTP (4). In turn, accumulating cGMP activates a cascade of intracellular signaling events resulting in the net secretion of fluid and electrolytes, which can manifest as diarrhea (6).

Beyond secretion, GUCY2C has emerged as a modulator of the dynamic crypt–villus and crypt–surface axes in small and large intestine, respectively (5). The GUCY2C hormone axis regulates the cell cycle and proliferation of transit amplifying cells in crypts, the size of the proliferating crypt compartment, DNA damage sensing and repair, the balance between glycolytic and mitochondrial oxidative metabolism, differentiation along the secretory lineage of epithelial cells, and epithelial–mesenchymal interactions underlying desmoplasia (7–11). These homeostatic processes regulated by GUCY2C organizing the crypt–surface axis also are the canonical processes universally corrupted in tumorigenesis (12). Indeed, genetic silencing of GUCY2C potentiates tumorigenesis in genetic and carcinogen-induced mouse models of colorectal cancer (7, 13). Conversely, enforced ligand activation of GUCY2C inhibits intestinal tumorigenesis in mice (14). Taken together, these observations establish that GUCY2C is an intestinal tumor suppressor with the potential to contribute to the initiation and progression of colorectal cancer (5, 14, 15).

In the context of GUCY2C as a tumor suppressor, preliminary studies suggest that guanylin expression is commonly lost in colorectal cancer (14, 16–20). In those studies, guanylin mRNA expression was substantially reduced in tumors compared with matched normal adjacent tissues (NAT; refs. 16, 18–20). Moreover, expression of this hormone also is uniformly lost in intestinal neoplasia in mouse models (14, 19). Guanylin loss in intestinal tumorigenesis in humans, and the conservation of this mechanism across species, suggests that colorectal cancer might initiate as a disease of paracrine hormone insufficiency, silencing the GUCY2C tumor suppressor, disrupting essential homeostatic mechanisms that alter the susceptibility of intestinal epithelial cells to transform (5, 14, 15, 21). The present study for the first time explores the expression of guanylin in primary tumors and matched NATs in a large cohort of patients with colorectal cancer, at mRNA and protein levels. This study establishes the near universality of guanylin loss, silencing the GUCY2C tumor suppressor, in colorectal cancer.

### Materials and Methods

#### Patients and tissues

For studies of mRNA expression, tissues were collected from patients participating in a prospective observational trial of the utility of GUCY2C quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in regional lymph nodes for staging patients with colorectal cancer (22). Between January 2003 and June 2007, we enrolled 313 patients with colorectal cancer at one of seven academic medical centers and two community hospitals in the United States and Canada (Fig. 1). Tumor specimens and/or normal mucosa were dissected from colon and rectal resections and frozen at −80°C within 1 hour of surgery to minimize warm ischemia. Of the 540 tissue samples, 440 tissue samples from 281 patients (29 with normal adjacent mucosa; 159 with matched tumors and normal adjacent mucosa; 93 with tumor only; Fig. 1) provided viable RNA quantified by the qRT-PCR of β-actin (ACTB), where
threshold cycles ($C_T$) were $< 40$ (22). Exclusions in this cohort included 6 patients with stage IV disease, 3 who had no clinical follow-up, and 23 who provided specimens yielding degraded RNA (Fig. 1). Disease status, obtained in routine follow-up by the treating physicians, was provided for all patients through December 2007. For studies of guanylin protein expression, tumors and NATs were obtained from deidentified archived formalin-fixed paraffin-embedded specimens formulated into tissue microarrays for immunohistochemical analysis (23). Protein expression was evaluated in adenocarcinomas ($n = 54$) and, where available, their matched normal epithelia ($n = 30$). Among these adenocarcinomas, 8% displayed muscular invasion, 23% displayed pericolic fat invasion, and 15% had metastasized to regional lymph nodes. Adenocarcinomas were well differentiated (24%), moderately differentiated (63%), or poorly differentiated (13%).

RNA isolation

RNA was extracted from tissues by a modification of the acid guanidinium thiocyanate–phenol–chloroform extraction method (24, 25). Briefly, individual tissues were pulverized in 1.0 mL Tri-Reagent (Molecular Research Center) with 12 to 14 sterile 2.5-mm zirconium beads in a bead mill (Biospec) for 1 to 2 minutes. Phase separation was performed with 0.1 mL bichloroethane, and the aqueous phase reextracted with 0.5 mL chloroform. RNA was precipitated with 50% isopropanol and washed with 70% ethanol. Air-dried RNA was dissolved in water, stored at $-80^\circ$C.

RT-PCR

The EZ RT-PCR kit (Applied Biosystems) was used to amplify target mRNA from total RNA in a 50-µL reaction. Optical strip-tubes were used for all reactions, which were conducted in an ABI 7000 Sequence Detection System (Applied Biosystems). In the kit components [50 mmol/L Bicine (pH 8.2), 115 mmol/L KOAc, 10 µmol/L EDTA, 60 mmol/L ROX, 8% glycerol, 3 mmol/L Mg(OAc)$_2$, 300 µmol/L each dATP, dCTP, and dGTP, 600 µmol/L dUTP, 0.5 U uracil N-glycosylase, and 5 U rTth DNA polymerase], the reaction mix contained target-specific forward and reverse primers and Taqman probe (Applied Biosystems), and 1 µg RNA template. The thermocycler program used for RT included: 50$^\circ$C for 2 minutes, 60$^\circ$C for 30 minutes, 95$^\circ$C for 5 minutes; and for PCR, 45 cycles of 94$^\circ$C for 20 seconds, 62$^\circ$C for 1 minute. Reactions were performed at least in duplicate and results were averaged.

Immunohistochemistry

Sections of tissue microarrays (23) representing formalin-fixed, paraffin-embedded normal epithelia and adenocarcinomas were deparaffinized and heated in a pH 9.0 antigen retrieval buffer (Dako) and processed in a Dako Autostainer using rabbit polyclonal anti-guanylin antibody (Sigma, Prestige Antibodies; catalogue #HPA018215) at a 1:60 dilution at RT for 30 minutes. Antigen visualization was performed using horseradish peroxidase (HRP)–conjugated anti-rabbit secondary antibody and diaminobenzidine as the chromogen.

Statistical methods

Target transcripts for guanylin were quantified relative to the intestinal epithelial cell marker villin (VIL) by qRT-PCR, using relative logistic regression models fit to fluorescence data from each well for replicate amplification reactions (26). Villin was chosen for normalization because it is a selective product of epithelial, compared with other cells, in intestine and is persistently and reliably expressed during tumorigenesis (27–29). Therefore, normalizing to villin permits estimates of epithelial cell-specific transcripts (e.g., guanylin) without tissue microdissection. For viable samples (ACTB qRT-PCR $C_T < 40$ cycles), target transcript expression was defined as the log relative expression ($R$) by:

$$\log(R) = m_R - m_T,$$

where $m_R$ and $m_T$ are estimated by the define of $m$ obtained from the logistic model (equation 1) for the reference gene (VIL) and the target gene (guanylin), respectively.

$$\Delta R(x) = L + \frac{L - L_T}{1 + e^{A_x}}$$

(1)

Each sample was analyzed at least in duplicate for each target gene for each tissue sample.

Standard paradigms for qRT-PCR analyses assign maximum $C_T$ values (e.g., $> 45$ cycles) to samples with undetectable target gene expression. However, this approach biases estimates of quantity and underestimates variance, inflating alpha levels in hypothesis testing. Here, analyses incorporated a multiple imputation algorithm, which selects random values from the lower tail of the distribution rather than assigning a fixed value of zero to samples with target gene expression below the lower limit of quantification (LOQ). This process, validated in studies quantifying HIV mRNA in plasma, limits bias in estimates of quantity and variance (30). In these analyses, 10 multiple imputations were implemented by SAS code (available on request) based on the likelihood of the truncated normal distribution (truncated at the LOQ), and those combined using Proc Mianalyze in SAS v 9.2.

Assessment of association of guanylin expression with clinical and demographic covariates was completed using linear mixed models based on the 10 imputed datasets as described above. Values of loss (NAT-Tumor) were computed on the basis of linear contrasts, with confidence intervals and $P$ values as estimated through Proc Mianalyze. Survival estimates were plotted using the Kaplan–Meier method, and differences estimated across strata based on the log-rank test. The association of NAT with NAT/Tumor was assessed via Spearman correlation.
### Results

#### Patient characteristics

Of the 281 patients providing viable specimens, 159 provided primary colorectal tumors and matched normal adjacent mucosa, whereas 29 patients provided only normal mucosa, and 93 provided only tumor (Fig. 1, Table 1). Clinicopathologic features, including depth of tumor penetration (T1/2, T3, T4), grade, and tumor anatomic location (right, left, sigmoid colon) were similar to national experience (31–33). There were no statistically significant differences in baseline characteristics of patients included and those excluded from analysis. Patients exhibited the well-established direct relationships between time to recurrence (\( P < 0.039 \)) and stage (Supplementary Fig. S1; refs. 31–33). Twenty-two percent of patients with pN0 and 76% with stage III colon cancer received adjuvant 5-fluorouracil-based chemotherapy.

#### Guanylin mRNA expression in normal mucosa

Across the population, basal guanylin expression varied more than a 100-fold range, reflecting interindividual variability in gene expression, rather than differential expression along the rostral–caudal axis (Figs. 2 and 3). Moreover, there is an inverse relationship between age and hormone expression reflected by a substantial decrease in basal expression of guanylin in patients older than 50 years (\( P < 0.01 \); Table 2). Basal expression of guanylin in normal epithelium was not significantly associated with tumor characteristics, including T, N, or American Joint Committee on Cancer (AJCC) stage (Table 2).

#### Guanylin mRNA expression in tumors

Profiling mRNA transcripts in small cohorts of patients suggested that guanylin expression is reduced during colon tumorigenesis (16–20). Indeed, guanylin mRNA expression was lower in tumors compared with NAT (\( P < 0.001 \); Fig. 2A–C; Table 2). In that context, 88% of tumors exhibited significantly attenuated expression of guanylin mRNA, compared with matched NAT, regardless of their anatomic site of origin (Figs. 2B and C; Table 2). The quantity of hormone loss was greatest in tumors arising from NAT with the highest guanylin expression (Spearman correlation coefficient = 0.61; \( P < 0.001 \); Fig. 2D). There was an inverse relationship between loss of guanylin expression in tumors and age, and patients ages <50 years exhibited the greatest loss, approximately 1,959-fold, whereas patients ages >50 years exhibited the least attenuation in expression, approximately 107-fold (\( P < 0.005 \); Table 2). As with basal hormone levels in normal tissue, attenuated expression of guanylin was not associated with tumor histopathologic characteristics, including T, N, or AJCC stage (Table 2).

#### Guanylin protein expression in tumors

The availability of reliable polyclonal antisera for immunohistochemistry of human tissues permitted interrogation of colorectal tumors and matched NATs for guanylin expression by tissue microarray analysis. Guanylin protein expression was strongly positive in all 30 specimens of normal epithelium (Fig. 4). Conversely, guanylin protein was absent in all 54 adenocarcinomas examined (Fig. 4). These immunohistochemical analyses confirm qRT-qPCR studies, which demonstrate near-universal attenuation of guanylin expression in colorectal tumorigenesis.

#### Discussion

Colorectal cancer is a disease of sequential accumulation of mutations in the single layer of epithelial cells lining the rostral–caudal axis of the large intestine (2, 3). Mutations in adenomatous polyposis coli (APC) and \( \beta \)-catenin, which are present in nearly 100% of tumors, lead to loss of normal growth control and the formation of small polyps (2). Further mutations in genes, including KRAS and BRAF, lead to exuberant hyperproliferation
and the formation of large premalignant adenomas (2, 3). Terminal mutations in p53, or the TGF\(\beta\) signaling components SMAD4 or the TGF\(\beta\) receptor, drive transformation to invasive adenocarcinoma with metastatic potential (2, 3). Although this sequence of mutations is well defined, mechanisms underlying the susceptibility of intestinal epithelial cells to accumulate the mutations driving tumorigenesis remain undefined.

The intestinal epithelium comprises a highly dynamic structure organized as crypt-villus units in small intestine and crypt-surface units in the colorectum (34). In these continuously regenerating structures, proliferating transit amplifying cells arise from stem cells at the base and migrate up the wall of the crypt. With their continuous cycles of chromosomal replication and cell division, these cells depend on unceasing DNA damage sensing and repair. At a point along their migration, these cells are reprogrammed, halting proliferation and undergoing differentiation into the canonical cell lineages of the intestine, including enterocytes, goblet cells, enteroendocrine cells, and in small intestine Paneth cells at the base of the crypt. This shift from proliferation to differentiation reflects coordination of key homeostatic mechanisms, including reprogramming of metabolism from glycolysis to oxidative phosphorylation and reciprocal epithelial–mesenchymal signaling. Following differentiation, cells continue their migration to the tip of the villus in small intestine or the crypt-surface junction in the large intestine, where they undergo apoptosis and shed into the fecal stream. This tight choreography of homeostatic processes is central to maintaining the structural integrity of the intestine in the face of complete epithelial renewal every 3 to 5 days. GUCY2C has recently emerged as a key regulator of these essential homeostatic processes organizing the

Figure 2. Guanylin mRNA expression in NATs and tumors from the colorectum. A, distribution of guanylin mRNA expression in all 188 NAT and 252 tumors. B, distribution of guanylin mRNA expression in 159 matched NAT and tumor specimens. C, change in guanylin mRNA expression in 159 tumors, compared with their matched NATs. Red lines connect NATs with adjacent tumor specimens with decreased guanylin mRNA expression. Black lines connect NATs with adjacent tumor specimens with increased guanylin mRNA expression. D, relationship between basal guanylin expression in 159 NAT (X-axis) and loss of guanylin expression in associated tumors (NAT/tumor; Y-axis). Tissues were obtained and processed, mRNA extracted, and guanylin mRNA expression quantified by RT-PCR analysis as described.
intestinal epithelium. Activation of GUCY2C and accumulation of its downstream second messenger, cGMP, decreases key drivers and increases inhibitors of the cell cycle to maintain the size of the proliferating crypt compartment (7, 8, 10, 11, 13). Similarly, GUCY2C signaling drives DNA damage sensing and repair, maintaining DNA integrity to prevent accumulation of mutations in key oncogenes and tumor suppressors (8, 13). Furthermore, GUCY2C drives epithelial cell differentiation, specifically supporting the development of the secretory lineage including Paneth and goblet cells (8). In addition, GUCY2C signaling orchestrates metabolic reprogramming, from glycolysis characterizing proliferation to mitochondrial oxidative phosphorylation essential for differentiated cell function (13).

Beyond its role in maintaining epithelial homeostasis, GUCY2C has emerged as a tumor suppressor whose silencing induces dysfunction in canonical pathways underlying transformation. Silencing GUCY2C disrupts the DNA damage sensing and repair machinery, promoting mutations in key tumor suppressors, like APC, and oncogenes like β-catenin (8, 13). Also, attenuating the GUCY2C signaling corrupts normal mechanisms regulating proliferation, enhancing expression of the drivers, while reducing expression of inhibitors, of the cell cycle, expanding the proliferating crypt compartment (7, 8, 10, 11, 13). Conversely, there is a contraction of the differentiated epithelial cell compartment, with specific losses in cells of the secretory lineage, including goblet and Paneth cells (8). Furthermore, silencing GUCY2C and cGMP production blocks metabolic plasticity, imposing glycolytic programming across the entire crypt–surface axis that precisely mimics the Warburg metabolic phenotype pathognomonic of neoplasia (13). Finally, attenuating GUCY2C signaling reprograms bidirectional interactions between epithelial and mesenchymal compartments, creating maladaptive circuits that drive the formation of desmoplasia, a defining feature of tumorigenesis (35). Indeed, silencing the GUCY2C tumor suppressor and disrupting normal homeostatic mechanisms induces many of the hallmark pathways considered essential for cancer (12). In that context, attenuating GUCY2C signaling substantially amplifies the incidence and burden of intestinal tumorigenesis in mouse models of genetic or carcinogen-induced colorectal cancer (8, 13, 14).
Surprisingly, while GUCY2C functions as a tumor suppressor, its expression is universally preserved in primary and metastatic colorectal tumors. Indeed, most colorectal tumors overexpress GUCY2C 2- to 10-fold compared with NAT (36). This apparent paradox can best be appreciated in the context of changes in the expression of GUCY2C hormone during tumorigenesis. Preliminary studies in small samples of patients demonstrated that guanylin mRNA was one of the most commonly lost gene products in colorectal tumorigenesis (16, 18–20). Furthermore, this hormone was lost early in the process of tumorigenesis, at the premalignant adenoma stage of transformation (18). Moreover, elimination of guanylin mRNA expression during transformation was conserved across species, and colorectal tumors in mice also lose guanylin expression (14, 19).

These preliminary studies offer a potential explanation for the paradox of universal overexpression of the GUCY2C tumor suppressor in colorectal cancer. They suggest that silencing of this tumor suppressor during transformation is functional reflecting loss of guanylin, rather than oncogenic reflecting tumor suppressor mutations (5, 37). Studies here confirm this hypothesis in a large cohort of patients with colorectal cancer. They demonstrate that the expression of mRNA transcripts encoding guanylin is reduced, or lost, in >85% of tumors, and this occurs in all anatomic locations across the colorectum and in all disease stages. Moreover, these studies for the first time confirm guanylin mRNA loss at the protein level by immunohistochemistry, demonstrating that the expression of guanylin protein is completely eliminated in 100% of tumors examined, compared with NATs, in a separate validation cohort. Together, these observations support the suggestion that the functional silencing of the tumor suppressor GUCY2C through loss of paracrine hormone expression is a universal mechanistic step in sporadic colorectal carcinogenesis.

Although the loss of guanylin expression seems to be universally associated with intestinal neoplasia in animals...
and humans, molecular mechanisms underlying hormone loss remain unclear. To date, genetic or epigenetic mechanisms of hormone silencing have not emerged. Clearly, there are transcriptional or posttranscriptional mechanisms involved, reflected in the reduction in guanylin mRNA broadly across most tumors. Interestingly, in contrast with mRNA, which is reduced in most tumors, guanylin protein expression is completely lost in 100% of tumors, compared with NATs. This could reflect the greater sensitivity of qRT-PCR with its ability for near-single transcript resolution, compared with immunohistochemistry (36). Alternatively, this hormone might be silenced by independent mechanisms at the levels of transcription (mRNA) and translation (protein), creating reinforcing mechanisms that ultimately silence the GUCY2C tumor suppressor. These considerations highlight the importance of defining the molecular mechanisms silencing hormone expression, to determine their reversibility to prevent tumorigenesis.

The present study supports the working hypothesis that silencing the GUCY2C tumor suppressor by eliminating paracrine hormone expression contributes to the pathophysiology of colorectal cancer. Indeed, loss of guanylin and silencing GUCY2C early, and the resulting disruption of the cell cycle and DNA damage sensing and repair, may contribute to accumulation of mutations underlying the oncogenomic basis of tumorigenesis. In that context, it is tempting to speculate that the substantial reduction (>95%; see Table 2) in guanylin expression in normal epithelia in people age >50 years revealed here might contribute mechanistically to the established epidemiologic vulnerability of this population to colorectal cancer (1, 38). These mechanistic considerations suggest that colorectal cancer might initiate as a disease of paracrine hormone insufficiency (5, 37). Like other diseases of endocrine insufficiency reflecting hormone loss, but preservation of receptor expression, silencing GUCY2C might be prevented by therapeutic replacement of GUCY2C ligands. These translational considerations are underscored by the recent regulatory approval of the oral GUCY2C ligand linaclotide to treat patients with irritable bowel syndrome-constipation type (39), and the initiation of a clinical program to explore its utility in preventing colorectal transformation in humans (ClinicalTrials.gov Identifier:NCT01950403; ref. 40).

Disclosure of Potential Conflicts of Interest
T. Hyslop has ownership interest (including patents) in Targeted Diagnostics & Therapeutics, Inc. S.A. Waldman reports receiving a commercial research grant from and is a consultant/advisory board member for Targeted Diagnostics & Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Disclaimer
The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions relating to this study.

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Figure 4. Guanylin protein expression is lost in adenocarcinomas. Immunohistochemistry of representative cases illustrates the presence of guanylin in all normal epithelia (A and B), but its universal loss in adenocarcinomas (C and D). Brown chromogen is diaminobenzidine.
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