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Expression of Guanylin Is Downregulated in Mouse and Human Intestinal Adenomas

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Guanylin is a pro-secretory hormone that is expressed in intestinal epithelia. Previously, we mapped the guanylin gene to mouse and human chromosomal regions containing multiple intestinal tumor-modifying loci. Here, we investigate whether guanylin expression is downregulated in precancerous human and mouse intestinal adenomas and whether diminished guanylin expression increases adenoma susceptibility in an animal model of intestinal cancer, the multiple intestinal neoplasia (*Min*) mouse. *In situ* hybridization analysis indicated diminished guanylin expression in both mouse and human adenomas. Northern analysis of mouse intestinal tissues showed strain-specific levels of guanylin expression but no correlation with the resistance or susceptibility of each strain to adenoma formation. Similarly, cDNA sequence analysis indicated no inactivating mutations or polymorphisms common to either the high or low adenoma-risk groups. Nonetheless, we have shown that significant loss of guanylin RNA in adenomas of mouse and human is a marker of intestinal epithelial cell transformation.

Key Words: **adenomatous polyposis coli; guanylate cyclase C; guanylin; *Min;* multiple intestinal neoplasia.**

Salt and water transport within the gastrointestinal tract is a tightly regulated process. A hormone that likely influences this process is guanylin, a low-molecular-weight peptide that is secreted from the epithelial cell layer of the small and large intestine (1, 2). Once elaborated into the intestinal lumen, guanylin binds its receptor, guanylate cyclase C (GC-C), which is located within the brush border membrane of villous enterocytes. Binding of guanylin to GC-C initiates a signal transduction cascade that culminates in activation of the cystic fibrosis transmembrane conductance regulator (CFTR). Chloride and bicarbonate secretion follow, generating osmotic water movement into the intestinal lumen.

We have mapped the guanylin gene to mouse chromosome 4 and to the syntenic region of human chromosome 1p34–35, regions known to contain several tumor-modifying loci (3). Specifically, the Modifier of *Min* 1 (*Mom1*) locus and other tumor-modifier genes have been mapped to a similar region as guanylin on mouse chromosome 4 (4). *Mom1* influences the occurrence of adenomatous intestinal polyps within a well-characterized mouse model of heritable intestinal neoplasia (4), the multiple intestinal neoplasia (*Min*) mouse, which carries a mutation in the adenomatous polyposis coli (*mApc*) gene (5). In humans, germline mutation of the *APC* gene leads to familial adenomatous polyposis coli (FAP), an autosomal dominant syndrome that results in numerous colonic and extracolonic adenomas and eventually leads to adenocarcinoma (6, 7). The *Mom1* locus was initially identified in the mouse by noting the effect of various mouse backgrounds on adenoma onset and number; some genetic backgrounds were more resistant to adenoma formation while other backgrounds were more susceptible to adenoma formation (4). Several candidate genes, in particular phospholipase A2 (*Pla2*), have been implicated as *Mom1* but none have been conclusively proven (8–10).

Based on the expression pattern of guanylin in the intestine, its location within a chromosomal region known to contain modifiers of mouse intestinal trans-

Abbreviations used: APC, adenomatous polyposis coli; CFTR, cystic fibrosis transmembrane conductance regulator; FAP, familial adenomatous polyposis coli; GC-C, guanylate cyclase C; MIN, multiple intestinal neoplasia; UTR, untranslated region.

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formation and its loss in human colorectal tumors, we speculated that there was a role for guanylin in intestinal neoplasia and colorectal cancer. We first investigated the expression of guanylin in adenomas from mice and humans carrying one germline mutation of *mAPC/APC* to elucidate the role of guanylin in adenoma formation. In addition, we tested the hypothesis that guanylin itself could have a modifying effect on susceptibility to adenomas by determining the level of guanylin expression in ten strains of inbred mice that display differing susceptibility to tumor formation when carrying the *min* allele of *mAPC* and by characterizing sequence polymorphisms of guanylin in these mouse strains. Although we observed a dramatic decrease in guanylin RNA expression in adenomas from both human and mouse, expression level variation and sequence polymorphisms did not correlate with susceptibility to Min-induced tumor formation in the mouse.

MATERIALS AND METHODS

In situ hybridization. *Min* mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and human colonic tissue was obtained from patients undergoing intestinal resection for familial adenomatous polyposis coli according to an IRB-approved protocol, as previously described (11). Fresh tissues from mouse and human were fixed in 4% paraformaldehyde and then saturated in 30% sucrose before being embedded in M-1 embedding matrix (Lipshaw, Pittsburgh, PA) and snap frozen. Cryostat sections were cut at 10–12 μm, air-dried on Vectabond-coated slides (Vector Laboratories, Burlingame, CA), and fixed with paraformaldehyde. For *in situ* hybridization, prehybridization and hybridization were performed as described (12, 13). [^{35}S]rUTP-labeled antisense and sense riboprobes were prepared from either pCR2.1 mgg (full-length mouse guanylin cDNA) or pMON22305 (gift of Mark Currie) which contains the full-length human guanylin cDNA (2). Following hybridization and washing under stringent conditions (50% formamide at 50°C, and 0.1× standard saline citrate plus 0.1% sodium dodecyl sulfate at 60°C), the slides were dipped in NTB2 emulsion (Kodak, Rochester, NY), exposed for 1–3 weeks at 4°C and developed in D19 developer (Kodak). Sections were counterstained with hematoxylin and eosin and photographed under brightfield and darkfield illumination.

Northern analysis. All mouse strains were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed according to Institutional Animal Care and Use Committee guidelines. Mice were sacrificed by CO_2 asphyxiation; intestinal segments were removed and flushed with ice-cold phosphate-buffered saline and frozen immediately in liquid nitrogen. All animals were sacrificed during a two-hour period thereby avoiding differences in expression between animals due to circadian fluctuations in guanylin expression (14). Samples were stored at −80°C until processing. Total RNA was extracted from tissue using acid guanidine isothiocyanate-phenol-chloroform extraction as previously described (15). For Northern blots, total RNA (10.0 μg) was fractionated by electrophoresis in a 1.5% agarose/1.9% formaldehyde gel, transferred to a nylon membrane (MagnaGraph, MSI, Westboro, MA) by capillary action and crosslinked to the membrane with short wavelength UV light. A 2.2 kb genomic fragment containing the complete guanylin mouse gene was isolated and radiolabeled with [^{32}P]CTP by random primer DNA synthesis and blots were hybridized under stringent conditions as previously described (12). Northern blots previously hybridized with ^{32}P-labeled guanylin fragment were re-hybridized with a labeled oligonucleotide complementary to 18S ribosomal RNA to quantitate the relative amounts of total RNA loaded in each lane of the gels as previously described (16, 17). Visualization and quantitation of positive signals were accomplished with the PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

Genomic DNA sequence analysis. Genomic DNA was prepared from tail tissue, digested overnight with proteinase K (30 μg/ml) at 55°C in 100 mM Tris (pH 8.6), 200 mM NaCl, 5 mM EDTA and 0.1% SDS in a total volume of 500 μl, extracted once with a 1:1 ratio of phenol:chloroform (pH 8.0) and once with chloroform, precipitated with 500 μl isopropanol, washed with 70% ethanol, air dried and resuspended in 100 μl TE (pH 8.0). Exons 1, 2 and 3 and up to 100 nucleotides of surrounding intron sequences were amplified by PCR from genomic DNA with the following primer pairs (all primer sequences are shown below), MG.cDNA5′/EX1.FL.REV, EX2.FL.FOR/EX2.FL.REV and EX3.FL.FOR/MG.cDNA3′ respectively, using 1 μl template, 0.15 μM each dNTP, 0.1 μM each primer in 1× PCR buffer (Boehringer Mannheim) in a total volume of 10 μl using a denaturation step of 94°C × 90 sec, followed by 31 cycles of 94°C × 30 sec, 54°C × 30 sec, 72°C × 90 sec. The products were verified by electrophoresis on 3.0% agarose, purified using microcon-100 filters (Amicon) and sequenced with the same primers, using an ABI377 automated sequencer at the DNA Core Laboratory at the University of Cincinnati (http://www.molgen.uc.edu/dnacore/index.htm).

cDNA sequence analysis. RNA was prepared as described in Northern analysis section of this manuscript. cDNA was prepared by heating 5 μg total RNA together with 20 ng oligo-dT (Boehringer Mannheim) to 90°C for 5 minutes and cooling on ice. Extension was performed in 1× PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl), 5 mM $MgCl_2$, 1 mM each dNTP, with 1 μl RNAguard (Boehringer Mannheim) and 2 μl using SUPERSCRIPT II Rnase H$^-$ Reverse Transcriptase (Life Technologies) at 37°C for 2 hours. The volume was adjusted to 100 μl with dH_2O and overlaid with mineral oil. 10 μl aliquots were used as template for PCR using guanylin-specific primers MG.cDNA5′ and MG.cDNA3′ using a denaturation step of 94°C × 90 sec, followed by 31 cycles of 94°C × 30 sec, 54° × 30 sec, 72° × 90 sec. The products were verified by electrophoresis on 3% agarose, purified using microcon-100 filters (Amicon) and sequenced with an internal primer, PRIMER 1778.REV using an ABI377 automated sequencer by the DNA Core Laboratory at the University of Cincinnati. All primers used in sequencing genomic DNA and cDNA of mouse guanylin are as follows: MG.cDNA.5′, TTCTCTGCATTGCATACTGCTACCA; EX1.FL.REV, AAGATGCCCATCCCTCGTTTCAGG; EX2.FL.FOR, TGCCTACAGCCTGGCCTCAT; EX2.FL.REV, CTAGCCACACTAGCCAAGAGC; EX3.FL.FOR, TGGTGGCCGTCCCTCACCA; INTRON2.REV, GAAGAAGTCTTGGTGAGGGACGG; Primer 1788.REV, CGCTGTGGCAGGGCAATAGATGCTGAG; MG.cDNA.3′, GGTTAACATAGCCCTCAGGCAAG.

RESULTS

In Situ Hybridization of APC$^{+/-}$-Induced Adenomas

To determine the transcription levels of guanylin within cells of adenomatous tissue, guanylin RNA-specific riboprobes were hybridized to sections of *Min* mouse jejunum and human FAP colon (Fig. 1). Intestinal architecture is normal in these sections with the exception of the areas of adenoma formation characterized by the loss of villus formation in *Min* mouse sections and the presence of irregular hypercellular glands lined with atypical epithelial cells in human FAP colonic tissue. Importantly, non-transformed, *APC* heterozygous tissue is present in close proximity to presumably *APC* homozygous tissue in all sections, allowing for direct comparison of guanylin expression.

Histological examination of *Min* mouse intestine demonstrates the transition from non-transformed mucosal epithelia to well-developed adenoma (Fig. 1A). In the same tissue section, robust mouse guanylin expression is seen in normal epithelium of both the crypt and villus regions (Fig. 1B). Guanylin expression is largely absent, however, in the adjacent adenomatous tissue. Higher magnification shows a fairly well defined margin of mouse guanylin expression at the edge of the adenoma, but not within it (Fig. 1C). No hybridization is detected when tissue sections are incubated with a guanylin riboprobe transcribed in the sense direction (Fig. 1D). Similar findings were seen in multiple adenomas from several *Min* mice examined.

Loss of guanylin mRNA is also seen in human FAP colon sample examined by *in situ* hybridization. Typical results obtained from several human colonic samples are shown in Figs. 1E–1H. Transverse sections stained with hematoxylin and eosin show foci of early adenomatous transformation in the colonic epithelia and surrounding normal tissue (Figs. 1E and 1G). Hybridization with a human guanylin antisense riboprobe indicates that guanylin RNA expression is strong on the surface epithelia of the tissue surrounding the adenoma but expression is markedly decreased in areas of early adenomatous change (Figs. 1F and 1H).

Guanylin RNA Expression in Selected Mouse Strains

Intestinal segments from ten inbred mouse strains were harvested and RNA was extracted, blotted onto nylon membranes and hybridized with a genomic fragment containing the complete mouse guanylin gene as probe. Strains were chosen and classified as resistant or susceptible to adenoma formation according to the effect of the *Mom1* allele when the *Min* allele of *mApc* is present (4, 9). All strains used in Northern analysis do not have mutations at the *mApc* allele. Normalized guanylin expression was determined by hybridizing blots with an 18S RNA probe; typical results of guanylin and 18S hybridization are shown (Fig. 2A). A comparison of guanylin expression in all ten mouse strains (Fig. 2B) shows a consistent pattern of guanylin expression with generally increasing levels of expression from jejunum to colon. Guanylin signal intensity varies significantly from strain to strain. It is especially low in strains C58/J and CBA/J and strong in strains C57/J and C3H/J. Strains that are particularly vulnerable to Apc^{Min}-induced tumors show widely differing guanylin expression levels, as do strains from the adenoma-resistant group.

Guanylin Sequence in Selected Strains

Determination of guanylin gene and cDNA sequence from each of the 10 mouse strains was also performed. Primers annealing to flanking regions of the guanylin gene and to untranslated regions of guanylin cDNA from proximal jejunum were used to amplify and sequence coding regions. The sequence results were compared with the published sequence of guanylin (Genbank Accession number U60528/U09741), and with each other. The results showed no sequence differences between the strains in the 5′UTR, the 3′UTR, the immediate intron sequences between exons 1 and 2 and exons 2 and 3, or in the open reading frame. Thus, sequence analysis of guanylin suggests that sequence differences at the guanylin locus do not explain the susceptibility or resistance to *Min*-associated tumor formation in these mouse strains.

DISCUSSION

We hypothesized that guanylin may be involved in the initiation or progression of intestinal adenomas. The tissue expression pattern and chromosomal location of guanylin are consistent with its possible role as a tumor-modifying gene. Therefore, we characterized the association of guanylin expression levels on susceptibility to intestinal tumor formation and the effect of adenomatous transformation on guanylin RNA expression. Our data suggest that constitutive RNA expression does not influence adenoma formation via the *Apc* pathway of mutagenesis, as there was no correlation between the levels of guanylin expression and the tumor number exhibited by ten mouse strains sensitive or resistant to the Apc^{Min} allele. Similarly, guanylin cDNA sequence analysis did not reveal any chain-terminating mutations or significant amino acid polymorphisms that would abolish or alter guanylin activity. However, it is notable that guanylin expression is greatly reduced in adenomatous cells within the intestine of *Min* mice and in humans with *FAP*. Recent studies of guanylin gene expression support this observation, showing that guanylin expression is drastically downregulated, albeit still present, in many human colorectal adenocarcinomas and human colorectal cancer cell lines (18). In addition, we have previously shown by *in situ* hybridization that guanylin expression is lost in human colorectal adenocarcinomas (19). Future experiments to address whether expression of guanylin in transformed cells would result in a lessening or loss of tumorigenicity will likely shed light on its role in neoplasia.

It remains unclear whether loss of guanylin expression is a cause or an effect of transformation and what mechanisms mediate low levels of guanylin expression. We have shown that significant reduction in guanylin levels occurs consistently in precancerous adenomatous tissue of mouse and human. Evidence suggests that loss of gene expression at this locus cannot be solely due to chromosomal alterations, that is, that loss of guanylin expression in *Min* mouse adenomas is not simply due to loss of genetic material at the guanylin locus (20). De-differentiation of transformed tissue

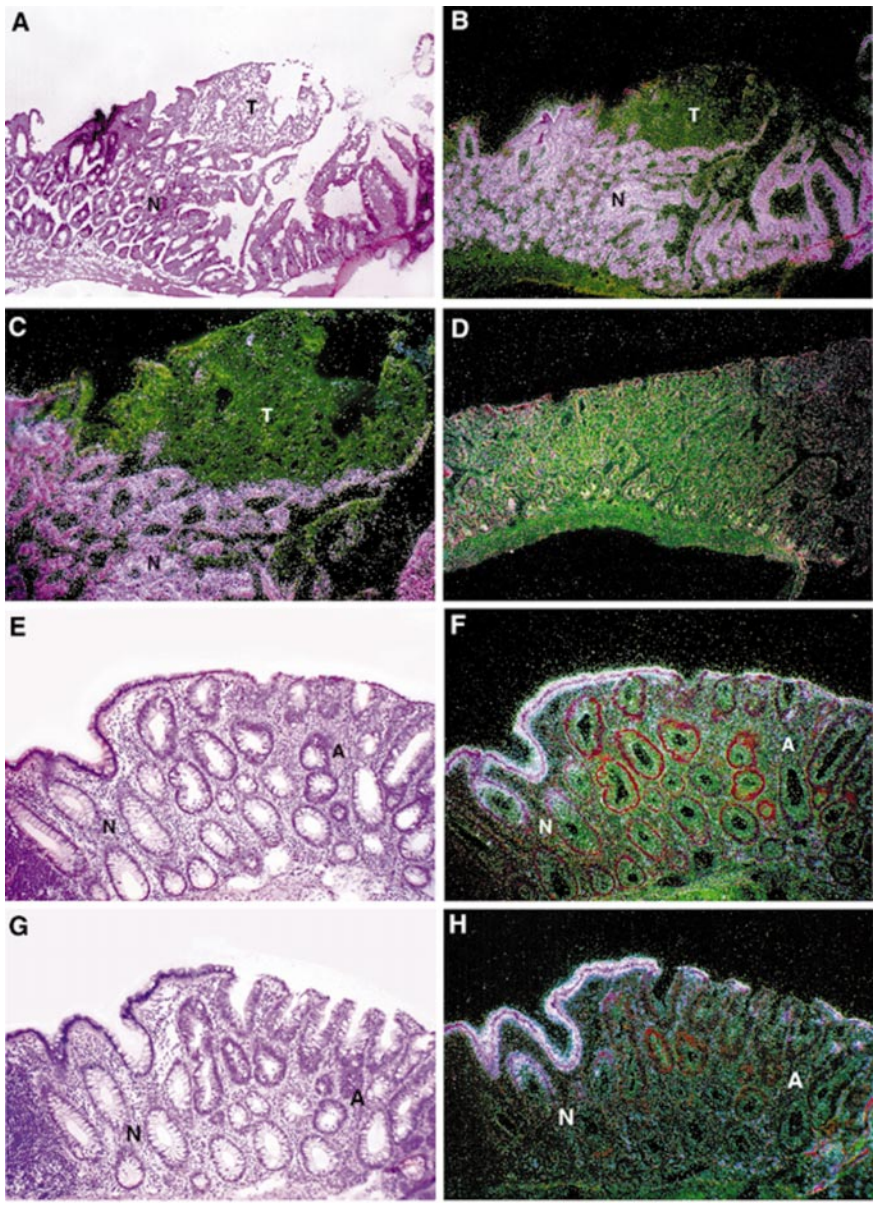

FIG. 1. *In situ* hybridization of guanylin riboprobe to mouse and human intestinal tissues. Mouse or human guanylin-specific riboprobes were hybridized to sections of *Min* mouse or human FAP intestinal tissue containing both normal (indicated by N (normal) in A–H) and transformed adenomatous cells (indicated by T (tumor) in A–D and A (early adenoma) in E–H). (A) Brightfield hematoxylin and eosin staining shows normal mouse intestinal structure as well as neoplastic tissue. (B) *In situ* hybridization of the same tissue section with a guanylin antisense probe indicates strong mouse guanylin signal in normal, nonadenomatous tissue and loss of any significant guanylin expression in the tumor. (C) A higher magnification darkfield image (400×) reveals a clearly defined tumor boundary that coincides with cessation of guanylin riboprobe signal. (D) A control hybridization indicates that a mouse guanylin sense probe does not bind specifically to any cells in the section (some autofluorescence can be seen in the base of the crypts and is due to Paneth cell granules). (E and G) Brightfield hematoxylin and eosin staining shows normal (N) human colonic tissue and regions of early adenomatous (A) change. (F and H) Strong guanylin expression is present on the surface epithelia of normal colonic epithelium while adjacent adenomatous cells show much less guanylin signal.

may result in loss of expression of genes typically associated with mature gastrointestinal cell types (21, 22). This may not be the sole cause of loss of guanylin expression, as significant levels of guanylin mRNA are present in immature colonocytes of the embryonic human and mouse, and are present in undifferentiated enterocytes of intestinal crypts (19).

Loss of guanylin expression may result from silencing of the guanylin locus as several mechanisms that inactivate transcription of guanylin are possible. Gene

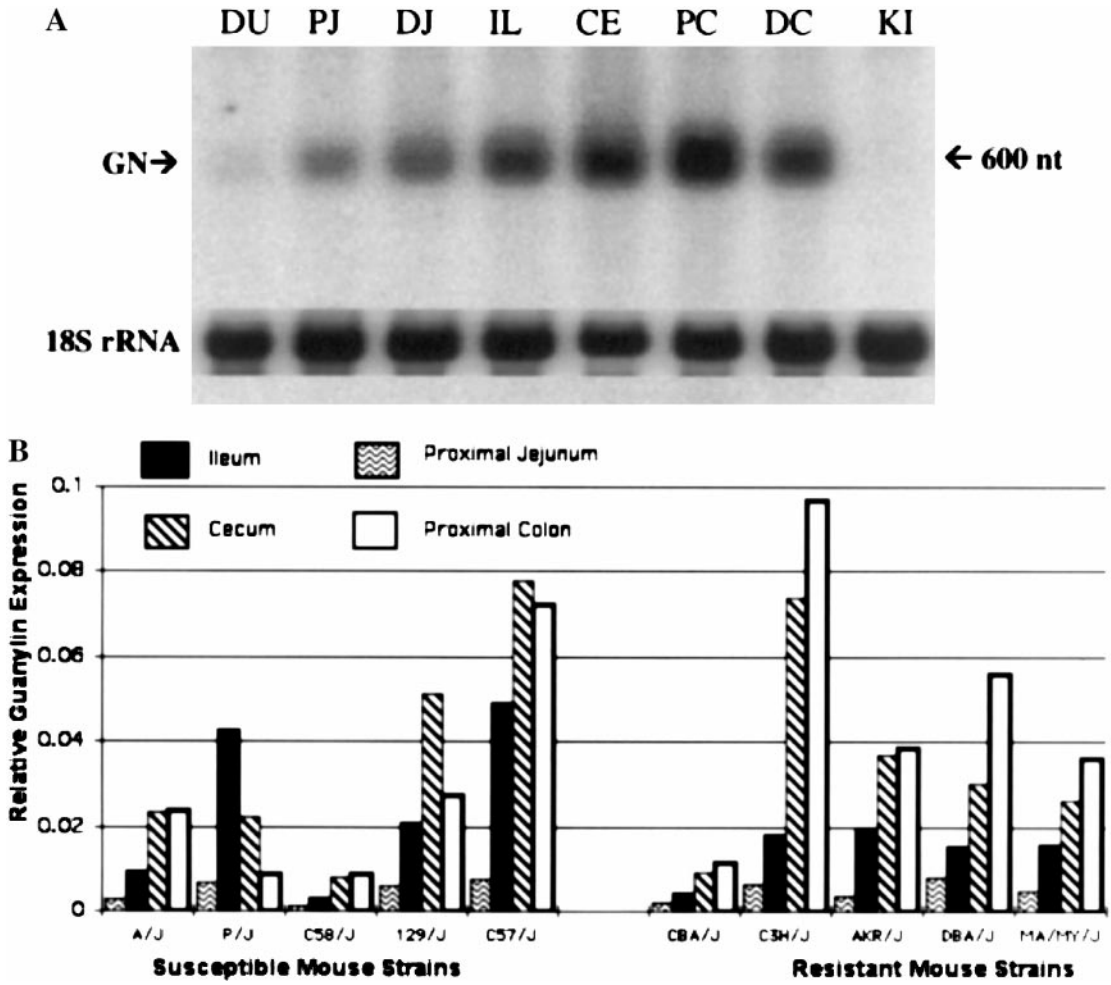

FIG. 2. Guanylin RNA expression levels in gastrointestinal segments from 10 inbred mouse strains. Blots containing 10 μg of RNA from gastrointestinal segments of 10 inbred mouse strains were hybridized with a probe specific for mouse guanylin. Signal was normalized to 18S ribosomal RNA levels. (A) A representative blot shows increasing guanylin RNA (600 nucleotides (nt)) expression from proximal to distal segments (DU, duodenum; PJ, proximal jejunum; DJ, distal jejunum; IL, ileum; CE, cecum; PC, proximal colon; DC, distal colon; KI, kidney) in the gastrointestinal tract of an MA/MY/J mouse. (B) A graphic representation of normalized guanylin expression levels of 10 inbred mouse strains (n = 2 mice per strain) is shown. The graph summarizes data for susceptible mouse strains (those that form large numbers of adenomas when the Apc^{Min} allele is present) and resistant mouse strains (those that form lower numbers of adenomas when the Apc^{Min} allele is present). Although guanylin RNA expression varies from strain to strain, there were no qualitative or quantitative differences in guanylin RNA expression between the susceptible and resistant groups as a whole.

silencing due to DNA hypermethylation has been demonstrated for other genes and may also influence guanylin expression in adenomas (23). This is especially relevant when considering that methylation levels may be considerably higher in adenomas as compared to normal mucosa (24). Guanylin downregulation may also be secondary to loss of necessary transcription factors that mediate activation at the guanylin locus. Examples include CDX1 and CDX2, caudal-related homeobox transcription factors whose expression is lost in colorectal cancers and have putative binding sites within the guanylin promoter (25–27). Transcription factors that influence guanylin transcription levels include HNF1-α, which is lost during renal cell carcinogenesis (28, 29). An attractive hypothesis is that, as in kidney epithelium, loss of HNF1-α expression occurs in intestinal epithelia during adenoma formation and this contributes to transcriptional silencing of the guanylin locus.

In contrast to guanylin, expression of the guanylin receptor, guanylate cyclase C (GC-C), is maintained in metastatic and in situ colorectal adenocarcinoma. Expression of this receptor is so invariate that it has been proposed as a tumor marker (30, 31). This suggests that paracrine secretion of guanylin from neighboring cells may still have an effect on transformed tissue within the intestine. However, since transformed tissue does not express guanylin but does consistently express GC-C, it may be possible to therapeutically target metastatic colorectal cancers using a guanylin-chemotherapeutic or radiopharmaceutical conjugate.

Our results indicate that guanylin RNA expression varies markedly in absolute levels between inbred strains. Similar to recently observed circadian regulation of guanylin in rats (14), these strain specific levels of guanylin expression are important to consider when designing future experiments. These differences in guanylin expression did not correlate with *Mom1*-associated resistance or susceptibility to adenoma formation. In addition, guanylin cDNA sequence analysis failed to demonstrate polymorphisms in guanylin cDNA from resistant or susceptible strains. However, guanylin mRNA transcript levels are strongly depressed in *Min* mouse and human adenomatous tissue when compared to surrounding normal tissue. These data indicate that while guanylin is not the major locus (*Mom1*) that modifies Apc^{Min}-mediated adenoma formation, its distinct loss in mouse and human adenomas is consistent with a role in neoplasia initiation and/or progression and suggests its potential use as a marker of intestinal epithelial transformation.

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