Sex Modulates Intestinal Transformation by the Tumor-Suppressor GCC

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Abstract

Background and Aims: Ovarian hormones oppose colorectal cancer, although mechanisms remain undefined. Similarly, the most commonly lost gene products in intestinal neoplasia include guanylin and uroguanylin, paracrine hormones for guanylyl cyclase C (GCC), which recently emerged as a tumor suppressor. However, the molecular interaction between intestinal paracrine and systemic sex hormones opposing intestinal neoplasia has not been explored.

Methods: Intestinal tumorigenesis was quantified in wild type (Gcc+/+) and GCC-deficient (Gcc−/−) mice carrying mutations in adenomatous polyposis coli (Apc) (Apcmin/+ or exposed to the carcinogen azoxymethane (AOM)). Proliferation of epithelial cells was examined employing cell cycle markers.

Results: Deletion of Gcc increased tumor multiplicity and growth in colons and small intestines, respectively, of Apcmin/+ mice. While changes in multiplicity and growth increased tumor burden, females exhibited approximately 60% (p = 0.040) of the burden in males. Similarly, female Gcc−/− mice treated with AOM exhibited approximately 40% (p = 0.048) of the burden in males. Moreover, Gcc deletion promoted epithelial cell proliferation, quantified by increases in β-catenin, cMyc, cyclin D1, and phosphorylated retinoblastoma protein (pRb), in males but not females.

Conclusion: There is a previously unappreciated interaction between sex and GCC signaling restricting crypt cell proliferation. Thus, the invariable loss of guanylin and uroguanylin resulting in tumorigenesis is mitigated in females by hormonal components of the ovarian axis. In the context of the universal overexpression of GCC by tumors, these observations highlight the combination of GCC paracrine and ovarian hormones for targeted prevention and therapy of colorectal cancer.

Keywords: colon cancer, sex, guanylyl cyclase C, proliferation, hormone deficiency, prevention, therapy

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer and cancer-related mortality in the world.1 Of all cases, approximately 20% reflect heritable genetic mutations, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), and these patients characteristically develop CRC as young adults.2-5 A majority (>80%) of cases are sporadic and the lifetime colon cancer risk in the general population is approximately 5%.6 Carcinogenesis reflects accumulation of multiple mutations in oncogenes or tumor suppressors, which transform colonocytes producing invasive carcinoma.7,8

There is an established relationship between the ovarian axis and colorectal tumorigenesis. The annual age-adjusted incidence of colorectal cancer in the United States is higher in men than in women.6,9 Also, there are sex differences regarding the anatomic location and clinicopathological characteristics of tumors.6,9 Women have a higher incidence of right-sided colon cancers compared to men.10-12 Interestingly, the clinical, histopathological, and molecular characteristics of colon tumors reflect the anatomic locations in which they arise.11-13 Left-sided tumors more frequently exhibit chromosomal instability (CIN) reflected by inactivation of tumor-suppressor genes, including adenomatous polyposis coli (APC) and p53, and mutations in the oncogene K-ras.6,14 In contrast, right-sided tumors are characterized by mutations in mismatch repair genes that underlie HNPCC.15,16 Unlike left-sided colon cancer, right-sided tumors exhibit microsatellite instability (MSI).15,16 Moreover, mechanisms underlying inactivation or mutation of tumor suppressors differ: DNA methylation and epigenetic changes, rather than loss of heterozygosity (LOH) in Apc and p53, are more frequent in right-sided colon cancers.17

Similarly, hormone replacement therapy (HRT) in postmenopausal women reduces the risk of colorectal cancer by approximately 30–40% and protects against the initiation and growth of adenomas, the precursor lesions of human colorectal cancers.18,19 In that context, activation of estrogen receptor β-suppressed proliferation of human colon cancer cell by controlling key cell cycle modulators, producing G1-S phase arrest.20 Moreover, overexpression of estrogen receptor α downregulated β-catenin and its downstream genes, including cyclin D1 and phosphorylated retinoblastoma protein (pRb), suppressing cell proliferation, and activated tumor necrosis factor, inducing apoptosis in human colon cancer cells.21,22 Furthermore, estrogen receptor α and β inhibited tumorigenesis in proximal colons of Apcmin/+ mice.23 These considerations highlight the potential of HRT to prevent and treat colorectal cancer.

Recently, colorectal cancer has been defined as a disease of localized hormonal deficiency.24 Indeed, the most commonly lost gene products in colorectal carcinogenesis include guanylin and uroguanylin, the endogenous ligands for guanylyl cyclase C (GCC),25-27 GCC and its ligands regulate intestinal epithelial cell dynamics and crypt-villus homeostasis by restricting cell proliferation28 through a transient arrest of the cell cycle.29 Moreover, elimination of GCC suppresses intestinal tumorigenesis in mice carrying mutations in Apc or exposed to the carcinogen azoxymethane (AOM) by restricting proliferation and maintaining genomic integrity.24,30 Further, oral administration of uroguanylin suppresses intestinal polypl formation and growth in Apcmin/+ mice.31

Here, the intersection of sex and GCC signaling opposing tumorigenesis, converging at restriction of crypt cell proliferation, was defined in mice carrying mutations in Apc or exposed to AOM. This previously unrecognized differential effect on tumorigenesis in males and females supports the hypothesis that GCC signaling plays multiple roles in opposing intestinal carcinogenesis beyond the restriction of cell proliferation. The
synergistic protection afforded by GCC signaling and female sex opposing colorectal cancer underscores the potential utility of combining GCC paracrine and ovarian hormones for targeted prevention and therapy of colorectal cancer.

**Material and Methods**

**Animal models**

Apc\(^{-/-}\)Gcc\(^{-/-}\) mice originally generated using I129/SvJ embryonic stem cells\(^1\) were backcrossed to the C57BL/6 strain. N\(_{11}\) (11th to 12th generation) mice were crossed to Apc\(^{Min}\) male and N\(_{11}\) mice were treated with AOM (Sigma, cat. A2853, St. Louis, MO, USA). All animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Apc\(^{Min}\)Gcc\(^{+/+}\) (male) and Apc\(^{Min}\)Gcc\(^{-/-}\) mice were established from Apc\(^{Min}\) (male) breeding founders to generate F2 Apc\(^{Min}\)Gcc\(^{+/+}\) (23 males and 9 females) and Apc\(^{Min}\)Gcc\(^{-/-}\) (15 males and 10 females) mice. The integrity of the Gcc gene was confirmed by genotype analysis and confirmed by analysis of \(^{213}\)I-ST binding in membranes harvested from small or large intestine.

**Immunoblot analysis**

Epithelia were dissected from normal intestinal mucosae from five male and three female Gcc\(^{+/+}\) or Gcc\(^{-/-}\) mice, protein extracted in Laemmli buffer containing protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL, USA), and extracts stored at −20°C. Extracts were subjected to immunoblot analyses employing antibodies to: β-catenin (cat. sc-7199), cMyc (cat. sc-764), cyclin D1 (cat. sc-718), and pRb (cat. sc-16671) from Santa Cruz (1:200 dilution, Santa Cruz, CA, USA) and GAPDH from Cell Signaling (cat. 2118, 1:1000 dilution, Danvers, MA, USA). Bovine anti-goat and anti-rabbit secondary antibodies were from Santa Cruz (1:5000 dilution, Santa Cruz, CA, USA). Staining intensity of specific bands quantified by densitometry was normalized to that for GAPDH. Average relative intensity reflects the mean of five male and three female mice.

**Statistical analysis**

Tumor multiplicity (number of tumors per animal) was analyzed by Poisson regression. Tumor burden and tumor size (mm\(^2\)) in the continuous scale were analyzed by linear mixed models, with random effect of animal to control for multiple measures per animal. Intensity of immunoblot staining was analyzed by \(t\)-test (2 tails) in both male and female groups.

**Results**

**GCC differentially suppresses intestinal tumorigenesis in male and female Apc\(^{Min}\) mice**

The impact of Gcc deletion on intestinal tumorigenesis was examined in male and female mice heterozygous for wild-type Apc. APC is mutated in >80% of sporadic colorectal tumors and germline mutations in APC underlie the inherited intestinal neoplastic syndrome FAP. Elimination of Gcc selectively increased tumor multiplicity in colon (Figure 1A) without altering tumor size (Figure 1B). Loss of Gcc significantly increased multiplicity in male and female Apc\(^{Min}\) mice, but with a lower magnitude in females. (B) Tumor size (diam\(^2\)) was quantified in male and female Apc\(^{Min}\)Gcc\(^{+/+}\) and Apc\(^{Min}\)Gcc\(^{-/-}\) mice. (C) Similarly, tumor number and size were quantified in small intestines of Apc\(^{Min}\)Gcc\(^{+/+}\) and Apc\(^{Min}\)Gcc\(^{-/-}\) mice. The size of tumors (diam\(^2\)) in small intestines was increased in male and female Apc\(^{Min}\)Gcc\(^{-/-}\), compared to Apc\(^{Min}\)Gcc\(^{+/+}\) mice. (D) Tumor multiplicity was modestly increased in both sexes in Apc\(^{Min}\)Gcc\(^{-/-}\) mice. (E) Tumor burden in each animal was calculated as the sum of the sizes of all tumors. Although tumor burden was significantly increased in male and female Apc\(^{Min}\)Gcc\(^{-/-}\) mice, it was significantly higher in males, compared to females. Bars represent mean ± SEM.

GCC differentially inhibits AOM-induced colorectal tumorigenesis in male and female mice

In mice, AOM mimics the induction of sporadic colon cancer in humans by inducing DNA alkylation-dependent base pair mutations, resulting in random single base alterations and DNA strand breaks.\(^2\) AOM-induced tumors were primarily restricted to the distal half of the colon. Similar to Apc\(^{Min}\) mice, deletion of Gcc significantly increased tumor multiplicity (Figure 2A) and growth (Figure 2B) in male and female mice. Tumor burden induced by AOM, reflecting increased tumor multiplicity and tumor size, was enhanced 3.6-fold in males (\(p = 0.01\)) and 2.3-fold in females (\(p = 0.06\)) by elimination of Gcc (Figure 2C). Tumor burden was significantly lower in female Gcc\(^{-/-}\), compared to male.
Gcc−/− mice (6.6 ± 1.5 mm² in male Gcc−/− mice vs. 3.0 ± 0.7 mm² in female Gcc−/− mice, p = 0.048).

GCC inhibits enterocyte proliferation in male, but not female mice
Elimination of Gcc in male mice increased proliferation of intestinal epithelia,24,28 reflected by overexpression of β-catenin (Figure 3A and 3B; p = 0.01), a key regulator of cell proliferation that maintains intestinal epithelial cell renewal.4,15 Hyperproliferation by upregulating β-catenin in male Gcc−/− mice reflects the established role of cGMP in targeting that protein for proteosomal degradation.26 Acceleration of the cell cycle in Gcc−/− mice was associated with increase in the expression of β-catenin downstream targets,24,35 including cMyc and cyclin D1 (p = 0.04; Figure 3A and 3B). Furthermore, upregulation of cyclin D1 resulted in phosphorylation of Rb (p = 0.0003), accelerating the cell cycle. These data suggest that GCC suppressed tumorigenesis by restricting proliferation and antagonizing the Wnt/β-catenin signaling cascade.24,35,37,38 In contrast, elimination of Gcc did not affect cell proliferation in intestines of female mice (Figure 3C and 3D).

Discussion
Human colorectal cancer is a heterogeneous disease in which genetic mutations and environmental factors contribute to tumor initiation, promotion, growth, and malignant transformation.7,8 Sex is a major factor in human intestinal neoplasia, and in the United States, women have a lower age-adjusted incidence of colon cancer compared to men. Moreover, this phenomenon is recapitulated in ApcMin−/− mice. Indeed, although ApcMin−/− mice, unlike humans, develop tumors predominantly in small intestine, there is a lower incidence and multiplicity of colorectal adenomas in females compared to males.29 This study extended those previous observations beyond genetic models of intestinal neoplasia, demonstrating the impact of sex on intestinal tumorigenesis in a model of chemical carcinogenesis, in mice exposed to AOM. Abundant prospective and retrospective analyses have revealed the inverse relationship between HRT in postmenopausal women and risk of colon cancer.40,41 Further, women experiencing surgical menopause exhibit an increased incidence of colorectal adenoma compared to those undergoing menopause naturally.42 Taken together, these observations suggest the utility of HRT for colon cancer prevention.40,41

GCC has recently emerged as a novel tumor suppressor central to the initiation and promotion of colorectal cancer.24,30,42 The endogenous paracrine hormones, guanylin and uroguanylin, are gene products most frequently lost during tumorigenesis, which occurs early along the adenoma-carcinoma sequence.25-27 Conversely, oral administration of GCC ligand suppresses tumor multiplicity and size in intestine of ApcMin−/− mice.30 Further, activation of GCC signaling suppresses human colon cancer cell growth.42-44 Moreover, GCC regulates homeostasis along the intestinal crypt-villus axis by restricting proliferation, the cell cycle and crypt hyperplasia, mechanisms that are corrupted by dysregulation of GCC signaling, which directly underlies intestinal tumorigenesis.40 Observations here suggest that GCC signaling restricts proliferation by modulating the Wnt/β-catenin pathway, promoting PKG1β-dependent degradation of β-catenin and inhibiting its nuclear translocation, reducing expression of downstream targets, including key regulators of the cell cycle such as cMyc, cyclin D1, and phosphorylated Rb. These observations suggest that colorectal cancer may represent, in part, a condition of paracrine hormone insufficiency. In that context, they implicate a role for paracrine hormone supplementation to prevent and treat intestinal tumorigenesis.24,45

The present observations reveal for the first time an intersection between sex and the GCC-based paracrine hormone axis regulating crypt homeostasis. They suggest that female sex and GCC signaling converge at restriction of crypt cell proliferation, a key mechanism underlying tumorigenesis promoted by dysregulated GCC signaling.24,28 Indeed, elimination of GCC in males decreased expression of β-catenin and its downstream targets regulating the cell cycle, including cMyc, cyclin D1, and pRb, key mediators of proliferation,42-44 and these changes were associated with tumor initiation and promotion. In striking contrast, elimination of GCC was without affect on β-catenin and its downstream effectors in females, reflected by diminished tumorigenesis in models of genetic or chemical carcinogenesis. These observations suggest that the variable loss of guanylin and uroguanylin and the resultant downstream effects
on tumorigenesis through unrestricted proliferation and the associated acceleration of the cell cycle and crypt hypertrophy are mitigated by select, but unknown, mechanisms in females. Moreover, they underscore the multiplicity of mechanisms by which dysregulated GCC signaling influences tumorigenesis. Beyond proliferation, GCC regulates metabolic remodeling, epithelial-mesenchymal interactions, and genomic integrity, which likely contribute to tumorigenesis in female mice in which GCC signaling was eliminated. In the context of the established association between HRT and menopause, and the risk of colon cancer, and the defined effects of estrogen on proliferative regulators, it is likely that hormonal components of the ovarian axis mediate these protective effects, although a contribution of androgens to the promotion of tumorigenesis in males cannot be ruled out.

The previously unappreciated synergy between sex and the GCC signaling axis suggests a unique opportunity for cancer prevention and treatment. Expression of guanylin and uroguanylin is invariably lost early along the continuum of neoplastic transformation, and the role of GCC in restraining proliferation suggests that receptor dysregulation reflecting ligand insufficiency is a central mechanism contributing to colorectal tumorigenesis. However, ligand insufficiency is associated with receptor overexpression, and GCC mRNA and protein are universally increased in human colorectal tumor, compared to normal epithelial, cells rendering them highly specific targets for therapy. Taken together, in the context of the standard of care in which hormone deficiencies are treated by replacement, synergy between systemic female and intestinal paracrine hormones in antagonizing tumorigenizing underscores the potential for combination HRT and GCC ligand supplementation for targeted prevention and therapy in colorectal cancer.

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Synopsis

Sex Modulates Intestinal Transformation by the Tumor-Suppressor GCC

There is an established relationship between sex and colorectal tumorigenesis, and the annual age-adjusted incidence of colorectal cancer in the United States is higher in men than in women. In addition, there are sex differences regarding the anatomic location and clinicopathological characteristics of tumors. Moreover, hormone replacement therapy (HRT) in postmenopausal women reduces the risk of colorectal cancer by 30–40%. However, while ovarian hormones mitigate colorectal cancer risk by modifying genetic and environmental determinants, the precise mechanisms remain undefined. In that regard, the most commonly lost gene products in intestinal neoplasia include guanylin and uroguanylin, paracrine hormones for guanylyl cyclase C (GCC), an emergent tumor-suppressor regulating crypt hyperplasia, proliferation, and the cell cycle. Of note, the molecular intersection between intestinal paracrine and systemic sex hormones opposing intestinal neoplasia has not been explored. Here, the impact of GCC on tumorigenesis was examined in mice carrying mutations in Apc (Apc\textsuperscript{Min\textplus}) or exposed to azoxymethane (AOM), a model of genetic and chemical intestinal carcinogenesis. Proliferation of intestinal epithelial cells was quantified employing molecular markers of the cell cycle. While elimination of GCC expression increased tumor burden in Apc\textsuperscript{Min\textplus} and AOM-treated mice, females exhibited only approximately 50% of the burden observed in males. Of note, elimination of GCC expression promoted epithelial cell proliferation in males but not in females. These observations reveal an intersection between sex and the GCC-based paracrine hormone axis, converging at restriction of crypt cell proliferation. They suggest that the variable loss of guanylin and uroguanylin and the resultant downstream effects on tumorigenesis are mitigated by sex. In the context of the established association between HRT, menopause, and the risk of colon cancer, and the inhibition by estrogen of cell cycle regulators, it is presumed that hormonal components of the ovarian axis mediate these protective effects. This previously unappreciated synergy between sex and the GCC signaling axis suggests a unique opportunity for cancer prevention and treatment. Indeed, while expression of guanylin and uroguanylin is invariably lost early along the continuum of neoplastic transformation, GCC is universally overexpressed by human colorectal adenocarcinomas. In the context of the standard care in which hormone deficiencies are treated by replacement, the present observations underscore the potential for combining systemic sex and GCC paracrine hormone supplementation for targeted prevention and therapy in colorectal cancer.

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