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## Guanylate cyclase C as a target for prevention, detection, and therapy in colorectal cancer.

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## 30 **1.0 Abstract**

31 **Introduction:** Colorectal cancer remains the second leading cause of cancer death in the  
32 United States, and new strategies to prevent, detect, and treat the disease are needed. The  
33 receptor, guanylate cyclase C (GUCY2C), a tumor suppressor expressed by the intestinal  
34 epithelium, has emerged as a promising target.

35 **Areas Covered:** This review outlines the role of GUCY2C in tumorigenesis, and steps to  
36 translate GUCY2C-targeting schemes to the clinic. Endogenous GUCY2C-activating ligands  
37 disappear early in tumorigenesis, silencing its signaling axis and enabling transformation.  
38 Pre-clinical models support GUCY2C ligand supplementation as a novel disease prevention  
39 paradigm. With the recent FDA approval of the GUCY2C ligand, *linaclotide*, and two more  
40 synthetic ligands in the pipeline, this strategy can be tested in human trials. In addition to  
41 primary tumor prevention, we also review immunotherapies targeting GUCY2C expressed  
42 by metastatic lesions, and platforms using GUCY2C as a biomarker for detection and patient  
43 staging.

44 **Expert Commentary:** Results of the first GUCY2C targeting schemes in patients will become  
45 available in the coming years. The identification of GUCY2C ligand loss as a requirement for  
46 colorectal tumorigenesis has the potential to change the treatment paradigm from an  
47 irreversible disease of genetic mutation, to a treatable disease of ligand insufficiency.

48

## 49 **2.0 Current Challenges in Colorectal Cancer**

50 Colorectal cancer (CRC) remains the fourth most diagnosed cancer, and the second leading  
51 cause of cancer death in the United States [1]. Worldwide, it accounts for as many as 1.2  
52 million new cases and 600,000 deaths per year [2]. CRC incidence and mortality has  
53 declined since the 1980s, paralleling adoption of screening; however, available screening  
54 methods (e.g. fecal occult blood, flexible sigmoidoscopy, colonoscopy) vary in terms of  
55 sensitivity and specificity, risks, and evidence supporting their implementation.  
56 Unfortunately, no screening method has proven to reduce all-cause mortality [3].  
57 Colonoscopy has become the gold standard, enabling removal of dysplastic lesions before  
58 progression to cancer. Yet, sensitivity decreases for lesions <1 cm, and the adenoma  
59 detection rate and completeness of polyp removal varies between providers [4, 5]. Indeed,

60 the superiority of colonoscopy over other screening approaches recently was questioned  
61 [6].

62  
63 Complicating limitations of current screening tools, widespread and ill-defined risk factors  
64 for CRC make it difficult to develop screening guidelines. A fraction of new tumors arise in  
65 patients with known genetic syndromes (e.g. Lynch syndrome, familial adenomatous  
66 polyposis); however >90% of cases are thought to be sporadic [7]. Age and family history  
67 play a role, hence colonoscopy is indicated at age 50 for patients with average risk, and at  
68 age 40 for patients with a first degree relative diagnosed at a young age [2]. But other risk  
69 factors of unclear significance include high-fat diets, tobacco smoking, alcohol  
70 consumption, and body mass index [8, 9]. Patients with inflammatory conditions of the  
71 bowel, such as ulcerative colitis, are particularly predisposed to CRC [10], and several  
72 studies have suggested benefits from low dose non-steroidal anti-inflammatory drugs [11,  
73 12, 13]. However, a pathophysiological link between these risk factors and tumorigenesis  
74 remains unclear, delaying the development of disease prevention schemes.

75  
76 Despite progress in early detection and treatment, ~25% of patients present with late  
77 stage disease [14]. Many promising agents for metastatic disease have **become clinically**  
78 **available** (e.g. tyrosine kinase inhibitors, epidermal growth factor inhibitors, anti-  
79 angiogenesis agents, etc.), but the five-year survival rate for this population remains only  
80 11.7% [2, 14]. Hence, strategies for prevention, detection, and treatment of primary and  
81 metastatic CRC are needed.

### 82 83 **3.0 Genetic Basis of Colorectal Cancer**

84 CRCs develop slowly, often requiring over a decade to accumulate mutations required for  
85 epithelial transformation (providing a long window for detection). **The average tumor**  
86 **contains 90 different mutations [15], but despite this genetic heterogeneity, 70-80% of**  
87 **sporadic (non-hereditary) colorectal tumors arise by a series of mutations typically**  
88 **described as the adenoma-carcinoma sequence [7, 16].** Canonically, this sequence begins  
89 with an inactivating mutation of the *adenomatous polyposis coli* (*APC*) tumor suppressor  
90 gene. The APC protein normally inhibits the accumulation of  $\beta$ -catenin, the downstream

91 mediator of the Wnt signaling pathway. APC loss enables phosphorylation and aberrant  
92 translocation of  $\beta$ -catenin to the nucleus in the absence of the Wnt ligand, where it  
93 participates in oncogenic transcription (activation of c-MYC and cyclin D1) [17].  
94 Subsequent activating mutations of oncogenes (e.g. *KRAS*), and deactivating mutations of  
95 tumor suppressors (e.g. *TP53*) characterize the progression from normal epithelium, to  
96 adenoma, to carcinoma [7, 16]. About 15% of sporadic tumors arise by a different  
97 mechanism, characterized by dysfunction of DNA mismatch repair genes, such as *MLH1* and  
98 *MSH2*. Here, defective DNA repair permits accumulation of mutations in short repeated  
99 genetic sequences (microsatellite sequences), resulting in a microsatellite instability  
100 phenotype. Finally, a third tumorigenic pathway is characterized by aberrant gene silencing  
101 via CpG island methylation, an epigenetic phenomenon. These adenomas often harbor  
102 mutational activation of the *BRAF* oncogene, and exhibit a characteristic sessile serrated  
103 architecture [7, 16]. Many tumors contain elements of more than one pathway; for  
104 example, hypermethylation of the *MLH1* mismatch repair gene contributes to a large subset  
105 of microsatellite unstable tumors.

106  
107 By comparison, tumors arising from hereditary CRC syndromes occur less frequently (3-  
108 5% of cases), and arise from germline, rather than somatic, mutations in the  
109 aforementioned pathways [7, 16]. Hereditary non-polyposis CRC (HNPCC, or Lynch  
110 syndrome) occurs most commonly, and arises from mismatch repair gene mutation.  
111 Familial adenomatous polyposis (FAP) arises from germline mutations of *APC*, resulting in  
112 thousands of colonic polyps early in life and 100% risk of cancer by age 40.

#### 113 114 **4.0 GUCY2C as a Target in Colorectal Cancer**

115 Although a wealth of genetic and epigenetic changes have been associated with intestinal  
116 transformation, a common causative agent has yet to be found. Recent studies have defined  
117 a role for the intestinal surface receptor, guanylate cyclase C (GUCY2C), as a tumor  
118 suppressor involved in the earliest stages of transformation.

119  
120 GUCY2C belongs to the particulate guanylate cyclase class of receptors, and appears on the  
121 apical brush border of the intestinal epithelium [18]. Early studies defined its role in

122 regulating luminal secretion, specifically as the receptor for the bacterial heat-stable  
123 enterotoxin, ST, the causative agent of traveler's diarrhea [19]. ST binding to the  
124 extracellular domain of GUCY2C activates the intracellular catalytic domain, converting  
125 GTP to cyclic GMP [18]. This second messenger activates cGMP-dependent protein kinase II  
126 (PKGII), leading to downstream phosphorylation and activation events, including water  
127 and electrolyte secretion via the cystic fibrosis transmembrane conductance regulator  
128 (CFTR) [18]. Predictably, persons with activating or deactivating mutations of *GUCY2C*  
129 exhibit intestinal hyper- or hypo- secretory syndromes, respectively [20, 21]. Our  
130 understanding of GUCY2C-induced signaling has since expanded to include regulatory roles  
131 in epithelial renewal along the crypt-villus axis [22, 23], GI barrier integrity [24, 25], injury  
132 response [26, 27], and the gut-brain satiety axis [28, 29]. Importantly, GUCY2C is densely  
133 expressed throughout the intestine, and overexpressed by tumor tissue, features that can  
134 be exploited for diagnostic and therapeutic goals [30, 31, 32].

135

136 Endogenous GUCY2C ligands, the **peptides** guanylin and uroguanylin, are among the most  
137 commonly lost gene products in mouse models and human CRC [33, 34, 35]. In a study of  
138 300 patients, >85% of colorectal tumors exhibited disappearance of guanylin mRNA and  
139 protein compared to normal adjacent tissue [33]. This loss occurs early in intestinal  
140 transformation, suggesting that an intact GUCY2C signaling axis opposes tumorigenesis [23,  
141 36, 37]. Indeed, mice in which GUCY2C expression is eliminated (*Gucy2c<sup>-/-</sup>*) exhibit a  
142 tumorigenic phenotype, including epithelial dysfunction, DNA mutation, cellular  
143 proliferation and migration, and metabolic reprogramming (Figure 1) [24, 37].  
144 Interestingly, diet-induced obesity in mice also leads to guanylin loss and tumor formation,  
145 suggesting a mechanistic link between CRC and a well-described risk factor, obesity [28].  
146 Conversely, GUCY2C activating ligands and downstream mediators suppress oncogenic  
147 drivers (e.g. pRb, cyclin D1, B-catenin, pAKT) and increase tumor suppressors (e.g. p21,  
148 p27) [23, 25, 38, 39]. These findings underlie the *paracrine hormone hypothesis*, whereby  
149 CRC arises from an environment of **ligand** loss and functional GUCY2C inactivation. This  
150 pathophysiological paradigm could transform colon cancer from an irreversible disease of  
151 genetic origin, to a treatable disorder of **ligand** insufficiency. Recent FDA-approval of the

152 GUCY2C ligand, *linaclotide*, and the entrance of two others in the clinical pipeline, makes it  
153 feasible to test **ligand** supplementation for chemoprevention of CRC in humans.

154

## 155 **5.0 GUCY2C Agonists for Colorectal Cancer Prevention**

156 GUCY2C peptide agonists available for chemoprevention of primary colorectal tumors  
157 include the endogenous **peptides** guanylin and uroguanylin, bacterial diarrheagenic heat-  
158 stable enterotoxins (STs), and the synthetic peptides linaclotide, plecanatide, and  
159 dolcanatide. These ligands share structural homologies and conserved mechanisms of  
160 action through GUCY2C activation and downstream cGMP production.

161

### 162 *5.1 Endogenous Ligands*

163 Guanylin and uroguanylin, the endogenous ligands for GUCY2C in the intestine, were first  
164 described in the early 1990s [40, 41]. They are produced and stored as propeptides, and  
165 undergo processing to their mature 15-mer (guanylin) or 16-mer (uroguanylin) forms. The  
166 mature **peptides** are thought to act on GUCY2C in a paracrine fashion to maintain epithelial  
167 homeostasis and fluid secretion [18]. Uroguanylin also serves an endocrine role in gut-  
168 brain satiety signaling [28]. The **peptides** exhibit complimentary roles along the axis of the  
169 intestine, with maximal uroguanylin expression in the small intestine, and guanylin in the  
170 large intestine [42]. However, even after two decades of study, the cells of origin remain  
171 controversial, and may continue to evolve as we elucidate the multiple roles of the GUCY2C  
172 signaling axis [43, 44, 45, 46]. Interestingly, despite >50% sequence homology, uroguanylin  
173 is principally active in acidic pH and guanylin in basic pH, further reflecting regional  
174 specificity [47].

175

176 The disappearance of guanylin/uroguanylin early in colorectal tumorigenesis reflects a  
177 tumor suppressive function. Preclinical studies in mice have demonstrated the potential of  
178 therapeutic **ligand** replacement. For example, in *Apc<sup>min/+</sup>* mice (a CRC model) oral  
179 uroguanylin supplementation inhibited tumorigenesis [48]. In another model, mice  
180 genetically modified to overexpress guanylin were resistant to DSS-induced colitis [25].  
181 Further, it was recently shown that diet-induced obesity suppressed guanylin expression in  
182 mice, leading to tumorigenesis, and specific enforcement of guanylin expression prevented



183 obesity-related tumors [49]. In all of these studies, no adverse effects were observed over  
184 the lifetime of the mice.

185

## 186 5.2 Enterotoxins

187 Heat-stable enterotoxins (STs) are produced by several diarrheagenic bacteria, including  
188 enterotoxigenic *E. coli*, *K. pneumonia*, *V. cholera*, and *Y. enterocolitica* [18]. First described  
189 as GUCY2C agonists in 1990, STs include a family of peptides with a conserved C-terminal  
190 region [19]. Structurally similar to guanylin and uroguanylin, STs contain an additional  
191 disulfide bond, contributing to their canonical heat stability and increased receptor binding  
192 affinity [18]. Ligand-receptor binding activates GUCY2C, leading to CFTR-driven fluid and  
193 electrolyte transport into the intestinal lumen, manifesting as secretory diarrhea.  
194 Enterotoxigenic *E. coli* is endemic in developing countries with poor sanitation  
195 infrastructure. Interestingly, these regions have a lower incidence of CRC, which may  
196 reflect life-long exposure to STs, increased GUCY2C activation, and suppression of epithelial  
197 dysplasia [39, 50].

198

## 199 5.3 Synthetic Peptides

200 Synthetic peptides sharing homology with natural GUCY2C ligands target the secretory  
201 function of GUCY2C for therapeutic purposes. The first agent developed, *linaclotide*  
202 (Ironwood Pharmaceuticals, Inc., Cambridge, MA), is an ST analog approved by the FDA for  
203 the treatment of chronic idiopathic constipation (CIC) and constipation-predominant  
204 irritable bowel syndrome (IBS-C). *Linaclotide* binds GUCY2C, inducing cGMP accumulation  
205 and fluid secretion. Double-blind, placebo-controlled, phase III clinical trials were  
206 completed for patients with IBS-C (MCP-103-302 and LIN-MD-31) and CIC (MCP-103-303  
207 and LIN-MD-01) [51, 52, 53]. *Linaclotide* met all primary endpoints, significantly reducing  
208 abdominal symptoms and severity of constipation. No differences in serious adverse events  
209 were observed between *linaclotide* and placebo. The most commonly reported side effect  
210 was diarrhea, an effect predicted by its mechanism of action. New agents, *plecanatide* and  
211 *dolcanatide* (Synergy Pharmaceuticals Inc., New York, NY), are uroguanylin analogs with  
212 increased potency [54]. Like *linaclotide*, these agents agonize GUCY2C and stimulate cGMP  
213 production. They reduced disease severity (e.g. weight loss, inflammatory infiltrate,

214 destruction of crypt architecture) in pharmacologic and genetic murine models of colitis  
215 [54]. In a phase I trial of 72 healthy volunteers, up to 48.6 mg of *plecanatide* was safe and  
216 well-tolerated [55]. Currently, *plecanatide* is in phase III clinical trials for CIC and IBS-C  
217 [56].

218

219 Given their safety in human trials, these compounds could be used as oral-  
220 chemopreventive agents for CRC. In principle, exogenous GUCY2C ligand administration  
221 would reconstitute the tumor-suppressing GUCY2C signaling axis, preventing colorectal  
222 tumorigenesis. A phase I trial is underway to identify oral *linaclotide* dosing regimens that  
223 stimulate GUCY2C in the rectum. Study participants receive a single oral dose of *linaclotide*  
224 daily for 7 days, and then are assessed for increases in cGMP levels in rectal biopsy, as well  
225 as safety and tolerability (Linaclotide Acetate in Preventing Colorectal Cancer in Healthy  
226 Volunteers, clinicaltrials.gov NCT01950403).

227

## 228 **6.0 GUCY2C-Targeted Immunotherapies for Metastatic Colorectal Cancer**

229 While prevention of CRC remains the clinical ideal, therapeutic strategies for advanced  
230 disease are also needed. A growing body of literature endorses immunotherapy for cancer  
231 treatment. The immune system has a remarkable ability to suppress neoplastic  
232 proliferation, as demonstrated by heightened cancer risk in immunocompromised patients  
233 [57, 58]. In part, this risk reflects diminished immune control of oncogenic viruses (e.g.  
234 human herpes virus 8 and Kaposi sarcoma, hepatitis B and C viruses and liver cancer, or  
235 Epstein-Barr virus and Hodgkin's lymphoma) [58]; **however**, these patients also are  
236 predisposed to cancers without known infectious etiologies (e.g. melanoma, thyroid, and  
237 colorectal cancers) [58]. Instead, these are thought to arise from poor immune surveillance  
238 against cancer cells in tumors and the circulation. For example, the presence of  
239 lymphocytes in CRC tumors is associated with delayed metastasis and prolonged survival  
240 [59]. Tumor cells have a propensity to bypass or overcome these natural defense  
241 mechanisms, creating an unmet need for therapies that improve the immune response to  
242 cancer antigens (e.g. vaccines, adoptive T cell therapy) or target cancer cells directly (e.g.  
243 immunotoxins) [60, 61].

244

245 Effective CRC immunotherapies require antigenic targets that maximize immunogenicity  
246 and minimize autoimmunity. The most explored target, the glycoprotein carcinoembryonic  
247 antigen (CEA), is upregulated in CRC, but also appears in organs outside the GI tract,  
248 leading to **potential** autoimmunity and immunological tolerance [62, 63]. In contrast,  
249 GUCY2C has unique anatomic and biological characteristics **that appear to circumvent**  
250 **these issues**. GUCY2C is expressed by intestinal mucosa from the small bowel to the rectum,  
251 and is overexpressed in primary and metastatic colorectal neoplasms [30, 31, 32]. **Further,**  
252 **expression is largely restricted to the luminal aspect of the GI mucosa, and its extracellular**  
253 **domain is antigenically distinct from other members of the guanylate cyclase family found**  
254 **in other tissues [64, 65, 66]. Importantly, GUCY2C resides in an immune privileged**  
255 **compartment, with minimal exposure to the systemic immune response [64, 65, 66].**  
256 **Limited cross-talk between systemic and mucosal immune elements protects normal**  
257 **mucosa expressing GUCY2C from autoimmune toxicity, while also limiting systemic**  
258 **tolerance to the antigen [64, 65, 66].** These **advantages** have led to the exploration of  
259 several GUCY2C-targeted immunotherapeutic strategies (Figure 1).

260

### 261 *6.1 Vaccines*

262 Similar to the yearly-recommended flu vaccine, cancer vaccines stimulate the immune  
263 system to destroy cancer cells by targeting tumor-specific antigens, while also generating  
264 long-lasting immunity [60]. Viral vector vaccines, engineered to contain the genes for  
265 cancer antigens, enhance antitumor immunity by stimulating the expansion of adaptive  
266 immune system elements, namely Type 1 CD4<sup>+</sup> T-helper cells, cytotoxic CD8<sup>+</sup> T cells and  
267 antibodies [61]. This paradigm forms the basis for a GUCY2C-targeted vaccine, designed to  
268 **elicit** immune responses to metastatic CRC.

269

270 The first GUCY2C-specific vaccine incorporated replication-deficient type 5 recombinant  
271 adenovirus (Ad5) encoding the extracellular domain of GUCY2C (Ad5-GUCY2C) [64, 65, 66].  
272 In a murine pre-clinical proof-of-concept study, the vaccine stimulated a GUCY2C-specific  
273 CD8<sup>+</sup> cytotoxic T-cell response, which killed GUCY2C-expressing colon cancer cells.  
274 Remarkably, survival in mice with lung and liver metastases improved, **without signs of**  
275 **inflammatory bowel disease, organ or metabolic dysfunction, or autoimmune tissue**

276 **damage** [64]. Interestingly, the vaccine produced strong CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, and B-cell  
277 responses in *Gucy2c*<sup>-/-</sup> mice, but produced only a modest CD8<sup>+</sup> T-cell response in *Gucy2c*<sup>+/+</sup>  
278 mice, which was attributed to **GUCY2C-specific** CD4<sup>+</sup> T-cell tolerance [66]. **To overcome**  
279 **this, the vaccine was modified to include an immunogenic T-helper epitope from foreign**  
280 **protein** [66, 67]. This new vector reconstituted CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, and memory  
281 responses [66]. This was the first demonstration that selective CD4<sup>+</sup> T-cell tolerance blocks  
282 GUCY2C-specific immunity and memory responses. Importantly, this paradigm may extend  
283 to other antigens, including those in melanoma and breast cancer, suggesting that  
284 overcoming CD4<sup>+</sup> T-cell tolerance may be a requirement in many cancer vaccine  
285 approaches [66, 68, 69].

286

287 **Preliminary results were recently reported for a phase I clinical trial exploring the safety**  
288 **and immunogenicity of this vaccination scheme in stage I/II colon cancer patients**  
289 **([clinicaltrials.gov NCT01972737](https://clinicaltrials.gov/ct2/show/study/NCT01972737))**[70]. The vaccine is analogous to the murine vaccine, but  
290 encodes the human GUCY2C extracellular domain fused to the **T-helper epitope PAn DR**  
291 **Epitope** (Ad5-GUCY2C-PADRE). Preliminary findings are consistent with the pre-clinical  
292 studies, with patients responding to the vaccine by producing GUCY2C-specific CD8<sup>+</sup> T-cell  
293 and B-cell responses, but not a CD4<sup>+</sup> T-cell response, suggesting that selective CD4<sup>+</sup> T-cell  
294 tolerance governs GUCY2C-specific immune responses in humans, as well as mice [70].  
295 **Moreover, like preclinical studies, the vaccine did not induce GUCY2C-targeted toxicity in**  
296 **any GUCY2C-expressing tissue**. Importantly, these first findings in humans support Ad5-  
297 GUCY2C-PADRE as a promising therapeutic approach for patients with GUCY2C-expressing  
298 malignancies.

299

## 300 *6.2 Adoptive T Cell Therapies*

301 The past decade has witnessed remarkable progress in an immunotherapy approach  
302 known as adoptive cell therapy (ACT). Rather than employing a vaccine or other drug to  
303 induce an immune response within a patient, **this strategy employs ex vivo tissue culture to**  
304 **expand naturally-occurring immune effectors or create them *de novo* for administration to**  
305 **the patient** [71]. One approach involves boosting the activity of naturally occurring  
306 immune responses present in tumors, called tumor-infiltrating lymphocytes (TILs), which

307 are suppressed by the tumor microenvironment [61]. TILs can be isolated from patient  
308 tumors, activated and expanded *ex vivo*, and reintroduced to the patient, bypassing  
309 immunosuppressive elements. Another approach involves *ex vivo* genetic manipulation of  
310 peripheral blood lymphocytes to retarget them to tumors by expressing cancer-specific T-  
311 cell receptors (TCRs). Both TIL and TCR-gene transfer approaches have been efficacious in  
312 mouse models and humans with metastatic melanoma [72, 73, 74, 75, 76]. An ACT  
313 alternative approach employs chimeric antigen receptors (CARs). Here, T lymphocytes are  
314 modified to express an engineered receptor comprised of intracellular T-cell signaling  
315 motifs and an extracellular antibody domain that recognizes antigens in an MHC/HLA-  
316 independent fashion [77, 78]. CD19-targeted CAR-T cells have shown remarkable promise  
317 in the treatment of refractory leukemia in humans [79, 80, 81]. Because CARs can  
318 theoretically employ antibodies targeting any **cell surface** antigen, ACT approaches may be  
319 vastly expanded and personalized for other malignancies, including solid tumors.

320  
321 While efficacious for certain cancers, ACT has **had mixed results** in CRC patients. **A recent**  
322 **report demonstrated regression of lung metastases in a patient with colorectal cancer**  
323 **injected with TILs targeting mutant KRAS [82]. However, prior** trials of ACT targeting CEA  
324 and Her-2 resulted in adverse autoimmune effects, including death [83, 84]. In contrast,  
325 GUC2YC-targeted CAR-T cells may target metastatic CRC cells without destroying healthy  
326 tissue, given the anatomical compartmentalization of GUCY2C on the luminal aspect of the  
327 intestine, beyond access by CAR-T cells, which recognize native GUCY2C. As a proof-of-  
328 concept, CD8<sup>+</sup> T cells bearing CARs targeted to mouse GUCY2C lysed murine colon cancer  
329 cells, **eliminated colorectal cancer metastases**, and prolonged survival in a mouse model of  
330 metastatic CRC, without toxicity [85].

### 331 332 *6.3 GUCY2C-targeted Immunotoxins*

333 Antibodies offer several advantages as an immunotherapeutic tool, including  
334 immunomodulatory capacity, interference in ligand-receptor interactions, and relative ease  
335 of mass-production. Indeed, antibody-therapies are well-established in the clinic, with over  
336 50 FDA-approved therapeutics [86]. For example, the monoclonal antibody bevacizumab  
337 (Avastin), which targets the vascular endothelial growth factor pathway, is FDA-approved

338 as first line treatment for metastatic CRC. Others include cetuximab (Erbix) and  
339 panitumumab (Vectibix), antibodies which bind to the extracellular domain of the  
340 epidermal growth factor receptor, blocking ligand binding and tumorigenic signaling [87,  
341 88]. Still, these agents offer limited improvements in survival: bevacizumab was approved  
342 as a first line agent for metastatic CRC in 2004, but only increased median survival from 15  
343 to 20 months [87].

344 The next generation of antibody therapies, antibody-drug conjugates (ADCs) enable  
345 targeted delivery of cytotoxic agents to specific tissues [89, 90]. ADCs are engineered by  
346 linking a cytotoxin to a monoclonal antibody, facilitating targeting to cells expressing  
347 cancer antigens, endocytic uptake, and intracellular delivery of the toxic payload.  
348 Conceptually, the targeted nature of ADCs reduces systemic exposure, and endocytic  
349 uptake reduces drug resistance by P-glycoprotein efflux pump, two of the pitfalls of existing  
350 chemotherapeutics [89]. However, as a relatively new drug class, ADCs historically have  
351 proven difficult to optimize, and have been associated with significant side effects due to  
352 non-specific targeting [90]. For this reason, only two have achieved FDA approval,  
353 adotrastuzumab emtansine and brentuximab vedotin, although several others have entered  
354 clinical trials.

355  
356 Recently, a model GUCY2C-targeted ADC was devised, consisting of a GUCY2C antibody,  
357 ricin toxin payload, and cleavable disulfide linker (4-succinimidylloxycarbonyl- $\alpha$ -methyl- $\alpha$ -  
358 [2-pyridyldithio]- toluene; SMPT) [91]. The ADC specifically targeted GUCY2C, underwent  
359 endocytosis, trafficked to lysosomes, and delivered a toxic payload to colon cancer cells  
360 [91]. In mice with CRC lung metastases, the ADC prolonged survival without compromising  
361 normal tissue [91]. A subsequent phase I clinical trial was recently completed, examining a  
362 human IgG1 monoclonal antibody to GUCY2C conjugated via a protease-cleavable linker to  
363 monomethyl auristatin E, an anti-microtubule agent. The ADC (TAK-264) was tested for  
364 safety and tolerability in 41 patients with GUCY2C-expressing metastatic gastrointestinal  
365 disease. Four patients in the highest dose group experienced dose-limiting toxicity  
366 (neutropenia), but the safety profile was deemed manageable, and preliminary data  
367 suggest antitumor activity [92].

## 368 **7.0 GUCY2C as a Biomarker in Colorectal Cancer Detection**

369 Features that elevate GUCY2C as a target for immunotherapy (overexpression by tumors,  
370 limited expression outside the gastrointestinal tract [30, 31, 32]) also have value for cancer  
371 detection and staging. Disease stage remains a key prognostic and therapeutic factor in the  
372 management of patients with CRC [93]. Whereas the resection of tumors restricted to the  
373 bowel wall (stage II) is often curative, patients with metastasis of tumor cells to lymph  
374 nodes (stage III) experience recurrence rates of up to 50% with surgery alone [2]. Although  
375 adjuvant chemotherapy remains controversial at stage II, progression to stage III is an  
376 indication for chemotherapy, increasing survival as much as 15% [2, 94]. Unfortunately,  
377 traditional staging by histopathological examination of lymph node tissue remains  
378 insensitive, leading to missed metastases, patient under-staging, and inappropriate patient  
379 management. For example, less than 0.01% of available tissue is typically reviewed, and as  
380 many as 25% of supposedly lymph node-negative (pN0) patients die of disease recurrence  
381 [93], suggesting undetected metastatic cells.

382

### 383 *7.1 GUCY2C mRNA as a biomarker*

384 The expression profile of GUCY2C makes it uniquely suited for the staging of primary  
385 colorectal tumors and occult metastases [32, 95]. In a blinded multicenter prospective trial,  
386 2570 lymph nodes from 257 pN0 colorectal cancer patients were examined for GUCY2C  
387 mRNA by quantitative real-time PCR [96]. Patients were followed for 24 months, and the  
388 primary outcome measure was time to recurrence. Remarkably, 87% of patients  
389 considered stage II by traditional histopathological techniques were found to harbor occult  
390 metastases by GUCY2C molecular staging, correlating with earlier time to recurrence.  
391 Furthermore, qRT-PCR was used to stratify patients by tumor burden, based on the  
392 number of positive nodes and relative GUCY2C expression across nodes [97]. For the first  
393 time, it was shown that patients with greater occult tumor burden had a greater risk of  
394 recurrence, and this method could be used to stratify patients based on prognostic risk.  
395 Importantly, molecular staging by GUCY2C RT-PCR has been validated across multiple  
396 users and laboratories and may replace conventional histopathologic evaluation for staging  
397 and therapeutic decision making in colorectal cancer [98, 99, 100].

398



399 7.2 GUCY2C as a target for diagnostic imaging agents

400 Positron emission tomography has become a mainstay for staging CRC and monitoring  
401 treatment response [101]. This method capitalizes on the increased metabolic demand, and  
402 therefore increased glycolysis, by cancer cells. Cancer cells take up the glucose analog, 2-  
403 [<sup>18</sup>F]fluoro-2-deoxy-D-glucose (FDG) to a greater extent than surrounding normal tissue,  
404 allowing visualization by PET. However, glucose requirements by other tissues decreases  
405 specificity; false positives (due to inflammation, surgery, diverticulitis, etc.) lead to  
406 unnecessary follow-up colonoscopy or inappropriate staging [101]. Alternative imaging  
407 modalities using molecular targets, rather than metabolic patterns, may address these  
408 issues [102]. Targeting imaging probes to GUCY2C offers a sensitive means of detecting  
409 tumors derived from intestinal epithelium. Conjugates of radionuclides and GUCY2C  
410 ligands (e.g. ST, uroguanylin analogs) specifically target GUCY2C-expressing xenografts  
411 [103, 104]. These agents can be visualized with gamma camera scintigraphy, and  
412 accurately differentiate tumors of gastrointestinal origin from surrounding tissue [103,  
413 104]. Further, GUCY2C-directed antibodies accumulate in cells via clathrin-mediated  
414 endocytosis of the antibody-receptor complex, with the potential to amplify delivery of  
415 imaging agents or therapeutic cargo [91].

416

417 **8.0 Conclusion**

418 Despite improvements in CRC screening, incidence and mortality are among the highest of  
419 all cancers, and while the genetic basis has been well described, therapeutic targets remain  
420 elusive. The intestinal receptor GUCY2C has emerged as a target uniquely suited for  
421 prevention, therapy, and diagnostics. Its role as a tumor suppressor, inactivated by **ligand**  
422 loss early in tumorigenesis, suggests a novel disease prevention paradigm focused on  
423 GUCY2C ligand replacement. A clinical program is underway ultimately to test this strategy  
424 with the FDA-approved agent, *linaclotide*, and other promising agents are emerging.  
425 Further, its expression profile in the intestinal lumen and metastatic CRC tumors offers an  
426 ideal target for a rapidly expanding array of cancer immunotherapies, including vaccines,  
427 T-cell therapies, and antibody-drug conjugates. Finally, GUCY2C can be exploited as a  
428 sensitive biomarker for the detection and staging of CRC. Translation to the clinics is



429 underway on multiple fronts. Novel approaches targeting GUCY2C could revolutionize the  
430 treatment of CRC.

431

## 432 9.0 Expert Commentary

433

434 Cancer research remains an ever-changing field, with exciting advances in the past few  
435 decades that have shifted traditional treatment approaches. Preventative strategies are the  
436 clinical ideal and successes have been achieved for several neoplasms, such as the  
437 decreased incidence of gastric cancer following the identification and reduction of *H. pylori*  
438 infections [1]. Likewise, colonoscopy has reduced the incidence of colorectal cancers by  
439 eliminating lesions before they become invasive and metastatic.

440

441 While screening has reduced the incidence of colorectal cancer, it remains the fourth most  
442 diagnosed cancer, and the second leading cause of cancer death, with a 5-year survival  
443 <15% in metastatic disease. GUCY2C appears to play a pivotal role in epithelial  
444 homeostasis, including intestinal barrier integrity and obesity, known risk factors for colon  
445 cancer, suggesting novel molecular pathways that may be pharmacologically targetable.  
446 The revelation of GUCY2C ligand loss and receptor silencing early in tumorigenesis may  
447 have a transformative impact, supported by the exploration of multiple translational  
448 avenues. With regards to cancer prevention, reactivation of the GUCY2C tumor suppressor  
449 pathway with exogenous peptides has shown promise in pre-clinical models. Though  
450 initially formulated for the treatment of irritable bowel disease and chronic constipation,  
451 the translation of the FDA-approved GUCY2C ligand, *linaclotide*, to CRC is feasible, as safety  
452 and efficacy are already established. However, long-term effects of *linaclotide* and other  
453 synthetic GUCY2C ligands have not yet been defined and longitudinal chemoprevention  
454 trials are required.

455

456 In the context of CRC treatment, the identification of GUCY2C as a biomarker and cell-  
457 surface target of metastatic CRC cells may usher in new biologics and immunotherapies.  
458 GUCY2C-targeted vaccines and antibody-drug conjugates have advanced into clinical  
459 testing. Further, detection of GUCY2C mRNA in lymph nodes offers a sensitive means of

460 staging the disease, enabling more accurate identification of patients at risk for disease  
461 recurrence. Appropriate intervention in patients with previously unrecognized occult  
462 metastases may improve survival, especially as targeted therapeutics enter the clinic.

463  
464 Another area of interest and debate is the nature of cancer inception, and implications for  
465 targeting strategies. Traditionally, disease recurrence and treatment failure are thought to  
466 result from the inevitable acquisition of mutations and epigenetic changes that allow  
467 cancer cells to evade destruction [105]. However, evidence increasingly indicates the  
468 presence of “cancer stem cells”, a subpopulation of cancer cells with stem-like  
469 characteristics (e.g., tumorigenesis, self-renewal, and differentiation) that underlie  
470 metastasis, recurrence, and chemoresistance [106]. Identification and targeting of cancer  
471 stem cell markers could enhance CRC therapies. For example, a recent study demonstrated  
472 co-expression of CD133 and the breast cancer resistance protein (BCRP)/ATP-binding  
473 cassette subfamily G member 2 (ABCG2) by human colorectal tumors [107].  
474 Downregulation of ABCG2 inhibited self-renewal capabilities and enhanced  
475 chemotherapeutic effects in double-positive colon adenocarcinoma cells. Dual-therapies,  
476 potentially targeting a universal CRC marker like GUCY2C as well as a marker of the stem  
477 cell subpopulation may be a new translational avenue. As we better-characterize these  
478 neoplastic markers, therapeutic strategies will continue to evolve.

479

## 480 **10.0 Five Year View**

481 A large body of work across multiple laboratories supports the hypothesis that GUCY2C  
482 ligand loss is a necessary step in tumorigenesis. In the next five years, the molecular steps  
483 in this process likely will be defined, potentially leading to new clinical targets.  
484 Furthermore, results of the first trials translating GUCY2C-targeting schemes to the clinic  
485 will become available, including the effectiveness of GUCY2C ligand supplementation with  
486 *linaclotide*, a GUCY2C-targeted vaccine, a GUCY2C-targeted antibody-drug conjugate, and  
487 GUCY2C-targeted CAR-T cells. Additional GUCY2C ligands (dolcanatide and plecanatide)  
488 entering the pipeline will likely be explored for similar use as chemoprevention agents.  
489 Ultimately, the next five years should provide the first insights into the potential for  
490 GUCY2C-targeting to influence human colorectal cancer outcomes.

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**11.0 Key Issues**

- The gastrointestinal epithelial receptor, guanylate cyclase C (GUCY2C) has been described as a novel tumor suppressor and reliable biomarker of colorectal cancer.
- Endogenous GUCY2C ligand loss has been widely-described as an early step in colorectal tumorigenesis, suggesting a therapeutic strategy of **ligand** replacement for chemoprevention. The GUCY2C agonist *linaclotide* is FDA approved for other indications and a phase I clinical trial examining its use for colorectal cancer prevention is underway.
- GUCY2C is overexpressed in colorectal cancer metastases and several immunotherapies targeting GUCY2C are being explored, including adoptive T-cell therapy with GUCY2C-targeted CAR-T cells, a viral vector vaccine, and a GUCY2C-targeted antibody-drug conjugate. The latter two are currently in early human trials.
- Cancer staging and imaging strategies targeting GUCY2C also are being explored. GUCY2C mRNA is a sensitive biomarker of occult lymph node metastases, improving cancer detection and staging.

507 **References**

508 **Papers of interest (\*)**

509 **Papers of considerable interest (\*\*)**

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