disease at earlier stages. Such tests using biomarkers should have high sensitivity and specificity, while producing a low number of false-negative and false-positive results, to prevent subjecting healthy individuals to unnecessary colonoscopies. Colonoscopy offers significant improvements in the detection rates for CRC, but the diagnostic value of this is limited in relation to costs, risks, and inconvenience [5]. Non-invasive biomarkers have the potential to greatly enhance screening acceptance. Several non-invasive tests for detecting CRC are available, of which the fecal occult blood test (FOBT) is the most commonly used [6,7]. However, this test lacks sensitivity as well as specificity for screening an average risk population. Thus, novel CRC biomarkers that will further enhance the detection of the disease and trigger follow-up colonoscopies when necessary should be developed. In addition to such detection biomarkers, prognostic markers which can predict the likely course of the cancer, stratification markers which can predict the likely response to drugs prior to beginning treatment, and efficacy markers which can monitor the efficacy of drugs treatment may also reduce the mortality rate of CRC.

Table 2. Molecular biomarkers for the detection of CRC.

Clinical use	**Subjects**	**Types**	**Potential markers**
In use	Stool	Protein	Fecal hemoglobin
	Serum	Protein	CEA
		Carbohydrate	CA19.9
Clinical validation	Stool	DNA	K-*ras*
		DNA	*APC*
		DNA	L-DNA
		DNA	*p53*
	Serum	Protein	TIMP-1
Preclinical development	Serum	Protein	Spondin-2, DcR3, Trail-R2, Reg IV, MIC1
		Protein	PSME3
		Protein	NNMT
		Protein	CRMP-2
		Protein	SELDI (apolipoprotein C1, C3a-desArg, α1-antitrypsin, transferring)
		Protein	HNP 1-3
		Protein	MIF
		Protein	M-CSF
		Protein	M2-PK
		Protein	Prolactin
		Protein	CCSA-2, -3, -4
		Protein	MMP-9, -7
		Protein	Laminin
	Plasma	DNA	Septin 9
	WBC	DNA	5-gene panel (CDA, BANK1, BCNP1, MS4A1, MGC20553)

Recent advances in genomics and proteomics have contributed to our understanding of the natural history of cancers. Genomic techniques, such as DNA microarray analysis and proteomic methods, for example, 2-dimensional electrophoresis and mass spectrometry, are now commonly used to evaluate the expression profiles of genes and proteins in cancer cells, their surrounding tissues, and body

fluids [8]. Identification of genes and/or proteins that are characteristic of the development of cancer can potentially uncover biomarkers that will aid in the diagnosis of CRC. In this review, we will focus on potential non-biomarkers which have recently been discovered and non-invasive biomarkers which are currently being used in clinical settings (Table 2).

2. Fecal Markers

2.1. Fecal Hemoglobin

Stool-based detection of CRC is quite simple, inexpensive, and the least invasive method of screening available [9]. FOBT detecting hemoglobin enzymatically or immunologically is the most widely used screening modality for CRC and [10]. Enzymatic FOBT measures the peroxidase-like activity of hemoglobin originating from any source. Therefore, enzymatic FOBT is susceptible to bleeding from both colorectal and upper gastrointestinal tracts. In addition, the ingestion of certain foods (red meats, fruits and vegetables) and medicines (non-steroidal anti-inflammatory drugs) led also to false-positive results. Immunological FOBT using antibodies which specifically detect human hemoglobin is not impacted by plant peroxidase in the diet. An important limitation of the FOBT is the relatively poor sensitivity at detecting early-stage lesions. Low sensitivity of the FOBT for the detection of colorectal neoplasms is reported to be ~10% of adenomas and 40~85% of CRCs. In fact, randomized clinical trials indicated that FOBT is not very reliable and that it only reduces CRC mortality by 30% [3,11].

2.2. Genes and Epigenetic Markers

Cryptal cells (colonocytes) are shed into the fecal stream and provide informative materials that can be used to detect genes and epigenetic markers in feces [12]. Unlike fecal blood, cryptal cells are shed continuously. Furthermore, the shedding of cancer cells from CRC occurs more frequently than from normal colonic epithelium. Fecal cells including cancer cells can be assessed by analyzing DNA mutations for targets such as K-*ras*, *p53*, and adenomatous polyposis coli (*APC*), by analyzing epigenetic markers such as microsatellite instability (MSI), or by measuring unfragmented long-form DNA (L-DNA).

K-*ras*, encoding a Ras family protein, functions as a guanine nucleotide binding protein that is involved in a signal transduction pathway including the phosphatidylinositol-3-kinase and serine/threonine protein kinase B pathways [13]. K-*ras* mutations are found in 40–50% of sporadic colon cancers and adenomas [14]. In addition, K-*ras* mutations being present in aberrant crypt foci, which are putative pre-cancerous lesions, are reported to be found in 13–95% of CRC [15–18]. Thus, K-*ras* mutations might be an important early event in colorectal carcinogenesis.

p53 encoding a tumor suppressor protein which regulates the expression of genes involved in apoptosis, angiogenesis, the cell cycle and maintenance of the genome [19]. Approximately half of human cancers contain mutated *p53* genes, and 30–60% of CRCs have mutations in the gene [20]. The mutations depend on the stage, grade and location of the cancer. The mutations appear to be at relatively late stage of colorectal carcinogenesis, and altered p53 has only modest impact on the

outcome of CRC. Therefore, the relatively low mutation rate of p53 at early-stage limits the use in DNA-based detection of CRC.

APC protein is another tumor suppressor which assembles on a scaffold protein, axin, with β-catenin and glycogen synthase kinase 3β to coordinate the regulation of β-catenin signaling [21]. Inactivation of the APC protein is responsible for both inherited and sporadic types of CRC. Like K-*ras*, *APC* mutation appears to be an early-genetic event during the progression from adenoma to adenocarcinoma, suggesting its potential for use as a screening biomarker. However, unlike K-ras, the mutations are distributed throughout the coding region, thereby making it technically difficult and time consuming to detect all of the potential mutations during screening for CRC [22,23].

Microsatellites are stretches of short DNA sequences which contain a motif of 1–5 nucleotides with tandem repeats [24]. The most common microsatellite in human DNA is a dinucleotide repeat of cytosine and adenine. These tandem repeats occur throughout the human genome. MSI occurs when microsatellites undergo changes in length. MSI is observed in approximately 15% of the CRC tumors. Tumors with MSI have better prognosis than stage-matched tumors with stable microsatellite [25]. In sporadic CRC, MSI most commonly occurs due to epigenetic silencing of the DNA mismatch repair gene, *MLH1* [26]. There are several MSI markers, among which *BAT26* is probably the most widely used.

Shedding of cryptal cells is a normal consequence of exfoliation [27]. Untransformed cryptal cells are shed continuously from the colonic mucosa. These cells usually undergo apoptosis. Conversely, malignant CRC cells shed from tumor mass have a decreased rate of apoptosis relative to normal cryptal cells, which facilitate detection of intact genomic DNA (L-DNA) as a potential stool-based marker. Boynton [28] amplified six genomic fragments of different length from each of four different genetic loci (*APC*, *p53*, *BRCA1*, and *BRCA2*) using fecal specimens collected from 25 CRC patients and 77 controls. In this study, when a positive L-DNA was defined as >18 bands detected from a possible 24 bands (4 loci × 6 fragments), the specificity for CRC detection was 97% and the sensitivity was 57%.

A large population-based study revealed that a fecal DNA panel consisting of 21 mutations (three in the K-*ras* gene, 10 in the *APC* gene, and eight in the *p53* gene; the MSI markers of *BAT-26*; and L-DNA) detects a greater proportion of CRC than FOBT without compromising specificity [29]. In addition, the sensitivity of the fecal DNA panel was 52% for invasive CRC and 41% for invasive CRC plus adenomas with high-grade dysplasia, whereas that of the FOBT was 13% for the former and 14% for the latter. In subjects with negative findings on colonoscopy, the DNA panel had a specificity of 94%, whereas the FOBT had a specificity of 95%. The results of the study clearly indicate that the DNA panel has a greater sensitivity than the FOBT without reduced specificity.

3. Serum or Blood Markers

3.1. CEA

Carcinoembryonic antigen (CEA) is a high molecular weight glycoprotein belonging to the immunoglobulin superfamily. The carboxy-terminal of CEA contains a hydrophobic region which is modified to provide a glycosyl phosphatidylinositol link to the cell membrane. While the presence can

be determined in biopsy samples, it is usually identified in serum. This protein has been used for many years as a biomarker of CRC as well as cancers developing in other tissues [30]. High CEA levels are specifically associated with CRC progression, and increased levels of the marker are expected to fall following CRC surgery [31]. However, even in the absence of cancer, high CEA levels may also occur in response to inflammatory conditions, such as hepatitis, inflammatory bowel disease (IBD), pancreatitis, and obstructive pulmonary disease. In addition, CEA may not be elevated when CRC is at advanced stage. Thus, CEA does not provide sufficient sensitivity and reliability for the early detection of CRC. The potential value of the CEA test lies in its use to measure the course of the progression of cancer as a prognostic marker. CRC patients with higher CEA levels have poorer prognosis.

3.2. CA 19-9

Carbohydrate antigen (CA) 19-9, which is the second most investigated gastrointestinal tumor marker, is known to be a sialylated Lewis-a antigen [32]. CA 19-9 was originally defined by a monoclonal antibody produced by hybridoma prepared from the spleen cells of mice immunized with the human CRC cell line, SW 1116. Although CA 19-9 is the best marker available for pancreatic adenocarcinoma, CA 19-9 is less sensitive than CEA for CRC and also gives less information than CEA [33]. Other carbohydrate antigens, such as CA 50, CA 195, CA 242, CA M26, CA M25, CA M43 and CA 72-4, have also been evaluated extensively [34], but, due to their sensitivity, stage dependency and specificity, these antigens are not useful markers for the detection of CRC.

3.3. Tissue Inhibitor of Metalloproteinase Type 1

Tissue inhibitor of metalloproteinase type (TIMP)-1 is a multifunctional glycoprotein which inhibits most matrix metalloproteinases (MMPs). The total levels of TIMP-1 in patients with CRC are significantly greater when compared to that of healthy blood donors who have a very narrow range of plasma TIMP-1 levels [35,36]. More importantly, TIMP-1 is capable of being detected at early stages of CRC. Conversely, plasma levels of total TIMP-1 in patients with colonic adenomas, IBD or primary breast cancer, do not increase [37]. Preoperative TIMP-1 levels were proposed as a stage-independent prognostic biomarker for CRC in two independent studies [38,39]. The results of these studies, however, indicated that elevation of TIMP-1 was restricted to advanced stages of CRC. Additional studies are required to validate the use of TIMP-1 for both early diagnosis and evaluation of the prognosis of CRC.

3.4. Five-Serum-Marker Panel (Spondin-2, DcR3, Trail-R2, Reg IV, MIC 1)

Four serum biomarkers, spondin-2, tumor necrosis factor receptor superfamily member 6B (DcR3), TRAIL receptor 2 (TRAIL-R2) and Reg IV were recently evaluated in 600 serum samples. All four markers, as well as a fifth marker, macrophage inhibitory cytokine 1 (MIC1), were elevated in patients with CRC when compared to normal controls and patients with benign diseases. Additionally, this five-serum biomarker panel may have better sensitivity and specificity than CEA to improve the detection rate of early stage CRC.

3.5. Nicotinamide N-methyltransferase and Proteasome Activator Complex Subunit 3

To analyze 16 matched CRC and adjacent normal tissue samples, two-dimensional gel electrophoresis and mass spectrometry were used. Then proteins found to be elevated in cancer tissue were further validated with serum samples. Elevated levels of nicotinamide N-methyl-transferase (NNMT) and proteasome activator complex subunit 3 (PSME3), which are not predicted to be secreted, were found in serum from patients with CRC [40,41]. Validation studies using 109 CRC samples, 317 healthy control samples, and 87 samples from patients with benign large bowel diseases revealed that the diagnostic accuracy of PSME3 was similar to that of CEA, and that NNMT was better than CEA at detecting CRC.

3.6. Collapsin Response Mediator Protein-2

By analyzing the secretomes of 21 cancer cell lines derived from 12 cancer types, collapsin response mediator protein-2 (CRMP-2) was identified to be a potential CRC biomarker in the serums of 201 CRC patients and 210 healthy controls [42]. The use of CRMP-2 alone showed better sensitivity, but poorer specificity than CEA. Combined detection using CEA and CRMP-2 however produced better sensitivity (77%) and specificity (95%) than detection using either of these markers alone (43 and 61% sensitivity, respectively; 87 and 65% specificity, respectively). Thus, CRMP-2 might be a valuable serum marker when used in combination with CEA.

4. MicroRNA

For the past decade, the development of genomic technology has revolutionized modern biological research and drug discovery. Functional genomic analyses enable biologists to perform analysis of genetic events on a global scale and they have been widely used in gene discovery, biomarker determination, disease classification, and drug target identification. In this article, we provide an overview of the current and emerging tools involved in genomic studies, including expression arrays, microRNA (miRNA) arrays, array CGH, ChIP-on-chip, methylation arrays, mutation analysis, genome-wide association studies, proteomic analysis, integrated functional genomic analysis and related bioinformatic and biostatistical analyses [43].

Post-transcriptional regulation of gene expression by miRNA has recently attracted major interest among cancer researchers in relation to its involvement in cancer development. More than 1000 miRNAs are expressed in human cells, some tissue or cell type specific, others considered as house-keeping molecules. Functions and direct mRNA targets for some miRNAs have been relatively well studied over the last years. Every miRNA potentially regulates the expression of numerous protein-coding genes (tens to hundreds), but it has become increasingly clear that not all miRNAs are equally important; diverse high-throughput screenings of various systems have identified a limited number of key functional miRNAs over and over again. Particular miRNAs emerge as principal regulators that control major cell functions in various physiological and pathophysiological settings [44]. Altered miRNAs are reported in cancers of several tissues, including colon [45–47], liver [48], prostate [49], esophagus [50], brain [51], pancreas [52], breast [53], and chronic degenerative disease [54].

As to CRC, Dr. Nakagama's group [55,56] has demonstrated that Staphylococcal nuclease homology domain 1 (SND1), a component of RISC, is frequently up-regulated in human colon cancers and also chemically-induced colon cancers in animals, as well as in preneoplastic lesions of the colon. Overexpression of SND1 in colon cancer cells caused down-regulation of APC and activation of the Wnt signaling pathway as a consequence, without altering APC mRNA levels. Post-transcriptional regulation of gene expression by SND1 was suggested to be mediated by miRNA through the 3'-UTR containing the miRNA target sequence. As for the miRNA expression profile, miR-34a was among the list of down-regulated miRNA in human colon cancer, suggesting its tumor suppressive role in colon carcinogenesis. Expression of miR-34a is tightly regulated by p53, and ectopic expression of miR-34a in colon cancer cells causes remarkable reduction of cell proliferation and induces senescence-like phenotypes. miR-34a also down-regulates silent information regulator 1 (SIRT1), which is a class III histone deacetylase and known to be a negative regulator of p53 through the modulation of acetylation at K382 of p53, and participates in the positive feedback loop of the p53 tumor suppressor network. Other investigators [57] also suggested tumor suppressor functions of miR-34a, in part, through a SIRT1-p53 pathway. Recently, miRNA expression in CRC has been found to be associated with microsatellite instability (MSI) subgroups, including low MSI and HNPCC-associated cancers [58]. Thus, miRNA are potential diagnostic and prognostic markers (Table 3), as well as therapeutic targets for CRC.

Table 3. miRNA related to prognosis of cancer.

Cancers	miRNA	Authors and Ref. nos.
Lung cancer	hsa-let-7	[59]
	hsa-let-7a-2	[60]
	hsa-miR-155	
	hsa-miR-196a2	[61]
	hsa-miR-221	[62]
	hsa-let-7a	
	hsa-miR-137	
	hsa-miR-372	
	hsa-miR-182*	
	hsa-miR-21	[63]
Hepatocellular carcinoma	hsa-miR-125b	[64]
Breast cancer	hsa-miR-21	[65]
Gastric cancer	hsa-miR-21	[66]
Colorectal cancer	hsa-miR-21	[67]
	hsa-miR-106a	[68]
Head and neck cancer	hsa-miR-7d	[69]
	hsa-miR-205	
Pancreatic cancer	hsa-miR-21	[70]
Acute myelogenous leukemia	hsa-miR-181 family	[71]
Chronic lymphocytic leukemia	hsa-miR-1-miR-15a	[72]
Ovarian cancer	hsa-let-7a-3	[73]
Esophageal cancer	hsa-miR-103 / 107	[74]

5. Other Potential Biomarkers

Habermann demonstrated that C3a-desArg is present at significantly higher levels in serum from patients with colorectal adenomas and carcinomas than in serum from healthy individuals [75]. Specifically, in a blinded validation study (n = 59), the use of C3a-desArg alone predicted the presence of CRC with a sensitivity of 97% and a specificity of 96%.

Analyzing the protein profiles of colon cancer serum and protein profiles of CRC tumors demonstrated that human neutrophil peptides (HNP)-1, HNP-2 and HNP-3, also known as a-defensin-1, adefensin-2, and a-defensin-3, are up-regulated in CRC patients [76.77]. Indeed, the IINP1-3 level in the serum of 48 CRC patients and 42 normal controls was capable of identifying CRC with a sensitivity of 69% and a specificity of 100%.

The gene expression level of macrophage migration inhibitory factor (MIF) is elevated in CRC tissues, suggesting the use of the protein as a potential biomarker for CRC. In an analysis of serum samples of 129 patients with colon cancer and 53 healthy control subjects, the serum MIF level was found to be significantly increased in patients with CRC [78]. Although the specificity of MIF is not as high as that of CEA (90.6% *vs.* 100.0%), MIF is more sensitive during early cancer detection (47.3% *vs.* 29.5%), which suggests that MIF may be used as a diagnostic marker in CRC.

The serum levels of both macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor are significantly higher in CRC patients than in healthy subjects [79,80]. In addition, serum levels of M-CSF are more associated with lymph node metastasis than CEA and CA 19-9, which suggests that serum M-CSF elevation in CRC patients might help predict the risk of lymph node metastasis of this tumor. M-CSF may offer additional information to that presented by classic prognostic factors.

Prolactin that is synthesized by the anterior pituitary gland is a hormone with multiple biological actions and is elevated in patients with CRC. A study that evaluated 47 CRC patients and 51 healthy controls revealed that prolactin can predict CRC with a sensitivity and specificity of 77% and 98%, respectively [81].

M2-pyruvate kinase is an isoform of glycolytic enzyme pyruvate kinase. Although this protein is a cytosolic enzyme, it is liberated into circulation via an unknown mechanism. It is suggested that M2-pyruvate kinase is released into circulation from dying cancer cells. M2-pyruvate may thus be a useful marker for the detection of CRC. Two independent studies revealed that the use of M2-pyruvate kinase for the detection of CRC has a sensitivity of 48-58% and a specificity of 90–95%. Further, when combined with CEA, the sensitivity of M2-pyruvate increases without decreasing the specificity [82,83].

Recent evidence suggests that the assessment of epigenetic events is one of the most promising means of identifying biomarker candidates for the early detection of cancer. DNA methylation, in which cytosines within the palindromic dinucleotide 5'-CpG-3' sequence are methylated, shapes the chromatin structure of DNA according to its functional state [84,85]. The cancer genome is frequently characterized by hypermethylation of specific genes. Therefore, epigenomics AG has developed a blood test for CRC that is based on methylation of SEPT9, NGFR and TMEEF2 [86]. The evaluation study of this test, using free-floating DNA extracted from plasma samples of 133 CRC patients and 179 healthy controls, in the same age range, to determine the methylation levels, using restriction

enzyme-based qPCR, revealed that the biomarker with the highest performance was SEPT9, which was capable of detecting CRC with a specificity and sensitivity of 95% and 52%, respectively, when a cutoff of 0.011 μg/L of methylated SEPT9 DNA was used.

Gene expression patterns in the peripheral blood reflect changes that occur within the cells and tissues of the body [86]. Han [87] extracted total RNA from the white blood cells of peripheral blood and identified differentially regulated genes using a microarray. Specifically, they used a panel comprised of five genes including B-cell scaffold protein with ankyrin repeats 1 (BANKI), B-cell novel protein 1 (BCNPI), cytidine deaminase (CDS), FERM domain containing 3 (MGC20553), and membrane-spanning 4-domains, subfamily A, member 1 (MS4AI), to detect CRC. This test had a sensitivity of 88-94% and a specificity of 64–77%.

Three proteins, colon cancer-specific antigen (CCSA)-2, CCSA-3 and CCSA-4, have shown promise as markers for the detection of CRC. Using a cutoff value of 2 μg/mL for CCSA-3, both CRC and advanced adenoma were detected with 89% sensitivity and 82% specificity [88]. When CCSA-4 was used with a cutoff value of 0.3 μg/mL, the sensitivity and specificity was 85% and 91%, respectively. The use of CCSA-2 at a cutoff of 10.8 μg/mL had an overall specificity of 78% and sensitivity of 97% when used on separate individuals with advanced adenomas and CRC from normal, hyperplastic polyp, and adenoma populations [89].

Remodeling of the extracellular matrix is important in the development of epithelial malignancies, and several extracellular matrix proteins that can be liberated into circulation have been evaluated as potential biomarkers. The results of these evaluations have revealed that the serum levels of MMP9 and MMP7 depend on the presence of CRC [90,91]. In addition, serum laminin and MMP7 can be used as independent prognostic markers of CRC [91,92].

However, large scale clinical studies are required to refine and validate the diagnostic accuracy of the findings mentioned above.

6. Conclusions

Cancer biomarkers and characteristics of an ideal biomarker for CRC are discussed in this review, as well as technologies for their detection. The focus of this article is on the use of biomarkers for anticancer drug development and clinical applications, including determination of prognosis as well as monitoring of response to therapy. Types of biomarkers include serum/blood markers, fecal markers and miRNA. Currently, the FOBT is the only screening modality for CRC. DNA-based fecal markers are promising but are not widely used in clinical settings. In addition, a lack of sensitivity and specificity preclude the use of all existing serum markers for the early detection of CRC. CEA is used to monitor therapy in advanced CRC, and the pre-operative level of CEA is used to provide prognostic information. However, there is insufficient evidence for routine use of other classic serum markers such as carbohydrate antigens and TIMP-1. Therefore, large scale validation studies are required to evaluate the potential for the use of biomarkers that have recently been discovered through '–omics' technology. Within clinical research, oncology is expected to have the largest gains from biomarkers over the next five to ten years. Development of personalized medicine for cancer is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery and monitoring of diseases. A major challenge in development of cancer biomarkers will be the integration of proteomics with

genomics and metabolomics data and their functional interpretation in conjunction with clinical data and epidemiology [93].

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Colorectal cancer chemoprevention by 2 β-cyclodextrin inclusion compounds of auraptene and 4′-geranyloxyferulic acid

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The inhibitory effects of novel prodrugs, inclusion complexes of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (GOFA) and auraptene (AUR) with β-cyclodextrin (CD), on colon carcinogenesis were investigated using an azoxymethane (AOM)/dextran sodium sulfate (DSS) model. Male CD-1 (ICR) mice initiated with a single intraperitoneal injection of AOM (10 mg/kg body weight) were promoted by the addition of 1.5% (w/v) DSS to their drinking water for 7 days. They were then given a basal diet containing 2 dose levels (100 and 500 ppm) of GOFA/β-CD or AUR/β-CD for 15 weeks. At Week 18, the development of colonic adenocarcinoma was significantly inhibited by feeding with GOFA/β-CD at dose levels of 100 ppm (63% reduction in multiplicity, $p < 0.05$) and 500 ppm (83% reduction in the multiplicity, $p < 0.001$), when compared with the AOM/DSS group (multiplicity: 3.36 ± 3.34). In addition, feeding with 100 and 500 ppm ($p < 0.01$) of AUR/β-CD suppressed the development of colonic adenocarcinomas. The dietary administration with GOFA/β-CD and AUR/β-CD inhibited colonic inflammation and also modulated proliferation, apoptosis and the expression of several proinflammatory cytokines, such as nuclear factor-kappaB, tumor necrosis factor-α, Stat3, NF-E2-related factor 2, interleukin (IL)-6 and IL-1β, which were induced in the adenocarcinomas. Our findings indicate that GOFA/β-CD and AUR/β-CD, especially GOFA/β-CD, are therefore able to inhibit colitis-related colon carcinogenesis by modulating inflammation, proliferation and the expression of proinflammatory cytokines in mice.

There were ∼1 million new cases of colorectal cancer (CRC) in 2002 (9.4% of the total cancers).[1] Globally, the mortality of CRC was reported to be 655,000 deaths per year in 2005.[2] There is at least a 25-fold variation in the occurrence of CRC worldwide.[1] The highest rates of incidence are in North America, Australia/New Zealand, Western Europe and Japan, especially in Japanese men.[1] These large geographic differences for CRC are probably explained by differences in environmental exposures and lifestyles.

There are several types of pathogenesis of CRC.[3] Among them, inflammation is linked with CRC development.[4] The risk of CRC in patients with inflammatory bowel disease

Key words: β-cyclodextrin, 4′-geranyloxyferulic acid, auraptene, inclusion compounds, antitumor activity

Abbreviations: AOM: azoxymethane; AUR: auraptene; CDs: cyclodextrins; COX: cyclooxygenase; CRC: colorectal cancer; DSS: dextran sodium sulfate; dUTP: deoxyuridine triphosphate; GOFA: 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (4′-geranyloxy-ferulic acid); IBD: inflammatory bowel disease; IL: interleukin; iNOS: inducible nitric oxide synthase; NF-κB: nuclear factor-kappaB; Nrf2: NF-E2-related factor 2; TdT: terminal deoxynucleotidyl transferase; Tnf: tumor necrosis factor; TUNEL: TdT-mediated dUTP-biotin nick end labeling

Additional Supporting Information may be found in the online version of this article

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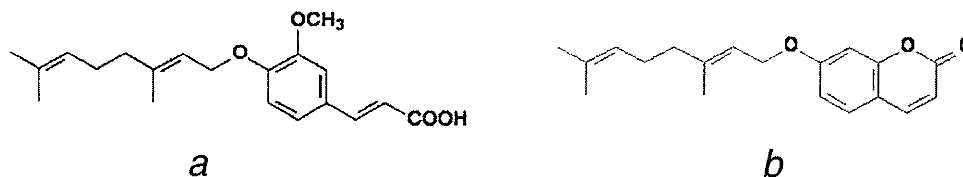

Figure 1. Chemical structures of (*a*) 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (GOFA) and (*b*) auraptene (AUR).

(IBD), including ulcerative colitis, increases with the increasing extent and duration of the disease.[3,5,6] A mouse model was recently established for colitis-related colon carcinogenesis[7] to facilitate the investigation of pathogenesis[8–10] and the chemoprevention[11,12] of inflammation-related CRC. In this mouse model of inflammation-related two-stage colon carcinogenesis, different types of colonic carcinogens can be used in combination with a colitis-inducing agent, such as dextran sodium sulfate (DSS), and many colonic tumors develop within a short-term period.[7,13–15] The powerful tumor-promoting effect of DSS may be due to the oxidative/nitrosative stress that is caused by DSS-induced colitis.[8–10] This suggests that the oxidative/nitrosative DNA damage associated with inflammation is involved in carcinogenesis, and, therefore, it is important to control the events that result in inflammation-related carcinogenesis.[16] In humans, the inflammatory cytokines and oxidative stress also play a key role in the pathogenesis of IBD-related intestinal damage.[17,18] As our understanding of the pathogenesis of IBD is currently inadequate, drug therapy of IBD and INB-related CRC has been empirical, *i.e.*, it is not based on a sound understanding of the etiology of the disease: drug therapy for IBD initially appears successful in the majority of IBD patients, and it comes with the risk of significant side effects. Therefore, we need new strategies, including chemoprevention, for IBD[19] and IBD-associated CRC.[3,20–23]

The natural and semisynthetic cyclodextrins (CDs) have been extensively studied to improve certain properties of the drugs, such as solubility, stability and bioavailability.[24] The CDs are suitable drug delivery systems because of their ability to greatly modify the physicochemical and biological properties of guest molecules through labile interactions by the formation of inclusion complexes. We have recently shown that modification of the physicochemical properties of violacein was achieved by the preparation of inclusion complexes with β-CD, thus leading to growth-inhibitory effects of its β-CD inclusion complexes against HL60 cells.[25–27] Many drugs currently used in the therapeutic management of colon diseases have been used as inclusion complexes with CDs.[28] The inclusion of the active principles in the cage represented by CD-protected drugs from absorption in the stomach and the upper portion of the lower intestine led to degradation of the saccharide portion in the large bowel by intestinal microflora, thereby ensuring a specific colon delivery with the maximum of bioavailability. This is the scope of the drugs that are being used in the therapy of malignant forms of colon cancer and IBD.

The 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (4′-geranyloxy-ferulic acid, GOFA) (Fig. 1*a*) is a prenyloxycinnamic acid that was extracted from the Australian small plant *Acronychia baueri* Schott (Family, Rutaceae) in 1966, and, in the last decade, was seen to exert valuable anticancer effects, particularly against tumors affecting the gastrointestinal apparatus.[23,29] Auraptene (AUR) (Fig. 1*b*) is a geranyloxycoumarin that is widespread in the natural kingdom and was extracted from plants belonging to several families (mainly Rutaceae and Apiaceae), comprising many edible fruits and vegetables, such as lemons, grapefruits and oranges. Like GOFA, AUR was seen in recent years to exert valuable pharmacological properties,[30] including dietary feeding colon cancer chemopreventive properties.[22]

As a continuation of our studies, we aimed to acquire further insights into the anticancer properties of selected prenyloxyphenylpropanoids. In our study, we wish to report the colon cancer chemopreventive activity of 2 novel prodrugs of GOFA/β-CD and AUR/β-CD that were obtained as their inclusion complexes with β-CD, using an inflammation-associated mouse colon carcinogenesis initiated with azoxymethane (AOM) and promoted by DSS.[7] For the mechanistic investigation of the effects of the 2 prodrugs on AOM/DSS-induced tumorigenesis, we determined the immunohistochemical expression of the proinflammatory cytokines, including nuclear factor-kappaB (NF-κB),[31–33] NF-E2-related factor 2 (Nrf2),[31,34] tumor necrosis factor (Tnf)-α[35–37] and STAT3[36] in adenocarcinomas that developed in the colon. In addition, the expression of interleukin (IL)-6[38,39] and IL-1β[40] was evaluated in the colonic epithelial malignancies. The effects of GOFA/β-CD and AUR/β-CD in the diet on cell proliferation and apoptosis of colonic adenocarcinomas were evaluated using proliferating cell nuclear antigen (PCNA)[21,41] for proliferative activity, apoptosis indices by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method[21] and positive rate of survivin[42] for apoptosis-inhibiting activity.

Material and Methods

Preparation of the inclusion complexes

GOFA and AUR were prepared according to previously reported methods.[43,44] β-CD was purchased from Aldrich Chemical. The inclusion complexes with a 1:1 molar ratio of

GOFA to β-CD and AUR to β-CD (113.5 mg, 0.1 mmol) were obtained by dissolving the geranyloxy derivative (0.1 mmol) in 100 mL of acetone, and soon thereafter, slowly evaporating the solution to dryness under vacuum in a rotatory evaporator at 45°C.[45] The structure of both inclusion compounds was determined by thermal analysis, X-ray diffraction, IR and NMR analysis, as already described.[46] The thermogravimetric analysis (TGA) data were obtained using a Polymer Laboratories (STA-625) thermal analyzer. The samples (2–6 mg) were heated in sealed aluminum pans under nitrogen flow (50 cm^3 min^{-1}) at a heating rate of 10°C min^{-1} from 50 to 500°C.[25,27,45,46] The powder X-ray diffraction patterns were recorded using a 6000-XRD (Shimadzu X-ray diffractometer) under the following conditions: Ni-filtered CuK radiation, voltage 40 kV, current 30 mA, at a scanning speed of 2° min^{-1} and count range 1,000 CPS. The detector was a proportional counter with a 1.7-kV detector voltage.[47–49] The samples of the solid dispersions and the physical mixtures of the complex and free drug and free CD were mixed with KBr and was pressed into a small tablet, which was mounted in the infrared beam. The spectra were recorded on the Perkin Elmer Model 1760X FTIR spectrometer from the KBr discs in the 500–4,000 cm^{-1} region.

Preclinical chemopreventive experiment

Animals, chemicals and diets. Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan), 5 weeks of age, were used in our study. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All the animals were housed in plastic cages (5 mice/cage) and had free access to tap water and a pelleted Charles River Formula (CRF)-1 basal diet (Oriental Yeast, Tokyo, Japan) during quarantine under controlled conditions of humidity (50 ± 10%), lightning (12-hr light/dark cycle) and temperature [(23 ± 2)°C]. They were quarantined for 7 days after arrival and randomized by body weight into the experimental and the control groups. A colonic carcinogen AOM was purchased from Sigma-Aldrich Chemical (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 Da (Lot no. 6046H) was purchased from MP Biomedicals, LLC (Aurora, OH). DSS for the induction of colitis was dissolved in water at 1.5% (w/v). β-CD inclusion complexes of GOFA (GOFA/β-CD) and AUR (AUR/β-CD) were synthesized, as described earlier. The experimental diets containing 0, 100 and 500 ppm of GOFA/β-CD (MW 1465.43) or AUR/β-CD (MW 1433.39) in a powdered basal diet CRF-1 were prepared weekly in our laboratory and stored in a cold room. The doses were selected based on our previous studies.[22,23] The animals had access to food and water at all times. The food cups were replenished daily with a fresh diet. All the handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

Experimental procedures. The Institutional Animal Care and Use Committee evaluated all the animal procedures that were associated with our study and assured that all the proposed methods were appropriate.

A total of 150 male ICR mice were divided into 5 experimental and control groups (Supporting Information Fig.). The mice in Groups 1–5 were initiated with AOM by a single intraperitoneal injection (10 mg/kg body weight). One week after the injection, 1.5% DSS (w/v) in drinking water was administrated to mice of Groups 1–5 for 7 days, followed by no further treatment for 18 weeks. The mice of Group 1 were maintained on the CRF-1 diet throughout the study. The mice of Groups 2 and 5 were fed CRF-1 diets containing 100 ppm GOFA/β-CD (Group 2), 500 ppm GOFA/β-CD (Group 3), 100 ppm AUR/β-CD (Group 4) and 500 ppm AUR/β-CD (Group 5) for 15 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 6 received AOM injection alone. Group 7 was treated with DSS alone. Groups 8 and 9 did not receive AOM and DSS and were fed CRF-1 diets containing 500 ppm GOFA/β-CD and AUR/β-CD, respectively. Group 10 did not receive any treatments and served as an untreated control. At the end of study (Week 18), all the mice were killed by CO_2 asphyxiation for careful necropsy, with emphasis on colon, liver, kidney, lung and heart.

At necropsy, the colons were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed with saline. They were cut and fixed in 10% buffered formalin for at least 24 hr. A histological examination was performed on the paraffin-embedded sections after hematoxylin and eosin (H&E) staining by one (T.T.) of the investigators. Colonic tumors were diagnosed according to Ward's description.[50] In brief, if the tumor cells with tubular formation invaded into the depth of the submucosa, the tumor was diagnosed as adenocarcinoma. When the tumor cells with glandular structure did not invade the submucosa and compressed the surrounding crypts, the tumor was diagnosed as adenoma.

Scoring of inflammation in the large bowel. Inflammation in the large bowel was scored on the H & E-stained sections made from all the mice. For scoring, the large intestinal inflammation was graded according to the following morphological criteria[51]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retention of the surface epithelium; Grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes and plasma cells) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry of NF-κB, Nrf2, Tnf-α, Stat3, IL-6, IL-1β, PCNA, TUNEL and survivin. The immunohistochemical analysis of the colon adenocarcinomas for the antibodies of NF-κB, Nrf2, Tnf-α, Stat3, IL-6, IL-1β, PCNA, TUNEL and survivin was performed on 4-μm-thick paraffin-embedded sections by applying the labeled streptavidin biotin method

using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections from the colonic neoplasms of the mice in each group ($n = 18$ in Group 1, $n = 11$ in Group 2, $n = 6$ in Group 3, $n = 6$ in Group 4 and $n = 3$ in Group 5) were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Tris-HCl buffer (0.05 M, pH 7.6) was used to prepare the solutions and was used for the washes between the various steps. The incubations were performed in a humidified chamber.

The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with primary antibodies. The primary antibodies included anti-NF-κB p50 (H-119) rabbit polyclonal antibody (#sc-7178, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit Nrf2 polyclonal antibody (#ab31163, 1:500 dilution; Abcam, Cambridge, MA), anti-human Tnf-α rabbit polyclonal antibody (#ab6671, 1:500 dilution; Abcam), anti-mouse Stat3 rabbit polyclonal antibody (#ab31370, 1:250 dilution; Abcam), anti-rabbit IL-6 polyclonal antibody (#ab6672, 1:400 dilution; Abcam), anti-mouse IL-1β rabbit polyclonal antibody (#LS-B40, 1:250 dilution; LifeSpan BioSciences, Seattle, WA), anti-rabbit survivin (71G4B7E) monoclonal antibody (#2808, 1:2,000 dilution; Cell Signaling Technology, Danvers, MA) and anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1,000 dilution; DakoCtytomation, Kyoto, Japan). These antibodies were applied to the sections according to the manufacturer's protocol. The horseradish peroxidase activity was visualized by the treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, the negative controls were performed on the serial sections without the first antibodies.

The levels of apoptosis in tumor tissues determined by the TUNEL method were done on 4-μm formalin-fixed, paraffin-embedded tissue sections of the colonic adenocarcinomas, according to the manufacturer's instructions using the Apoptosis *in situ* Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Osaka, Japan). The kit is based on the TUNEL procedure. The appropriate positive and negative controls for determining the specificity of staining were generated. The negative controls were processed in the absence of the TdT enzyme in the reaction buffer. Sections of tissue digested with nuclease enzyme and colon lymphoid nodules, which are known to exhibit high rates of apoptosis, were used as the positive controls. The color was developed with the peroxidase substrate 3,3′-diaminobenzidine and the sections were counterstained with Mayer's hematoxylin (Merck).

Immunohistochemical evaluation and scoring. The immunoreactivity against the antibodies, except PCNA, TUNEL and survivin, was assessed in the large colonic adenocarcinomas (more than 3 mm in diameter) developed in Groups 1–5 using a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). The intensity and localization of the immunoreactivity against the primary antibodies were determined by a pathologist (T.T.) who was unaware of the treatment group to which the slide belonged. The immunoreactivity was evaluated against the NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β antibodies with grading between 0 and 5: 0 (~15% of the colonic cancer cells showing positive reactivity), 1 (16–30% of the colonic cancer cells showing positive reactivity), 2 (31–45% ofthe colonic cancer cells showing positive reactivity), 3 (46–60% of the colonic cancer cells presenting positive reactivity), 4 (61–75% of the colonic cancer cells showing positive reactivity) and 5 (~75% of the colonic cancer cells showing positive reactivity).

The number of nuclei with positive reactivity for PCNA-, TUNEL- and survivin-immunohistochemistry was counted in a total of 3 × 100 cells in 3 different areas of the colonic cancer and expressed as a percentage (mean ± SD).

Statistical evaluation. Where applicable, the data were analyzed using 1-way ANOVA with Tukey-Kramer Multiple Comparisons Test (GraphPad Instat version 3.05, GraphPad Softwear, San Diego, CA) with $p < 0.05$ as the criterion of significance. The Fisher's exact probability test was used for comparison of the incidence of lesions between the 2 groups.

Results

General observation

During the experiment, a few animals of Groups 1–5 and 7 (DSS alone) had bloody stool, but the symptom disappeared soon after stopping the DSS treatment. At Week 18, some of the mice of Groups 1–5 had bloody stool again and anal prolapse because of rectal tumor. The mice belonging to Groups 6 (AOM alone), 8 (GOFA/β-CD alone), 9 (AUR/β-CD alone) and 10 (untreated) did not have any symptoms related to the treatments during the experimental period. As summarized in the Supporting Information Table, there was no significant change between the experimental groups with respect to the parameters tested (body and spleen weights). The liver and relative liver weights of Groups 6 and 8 were significantly smaller in comparison to Group 10. With respect to colon length, the value of Group 1 was significantly lower in comparison to Groups 6 ($p < 0.05$) and 7 ($p < 0.05$). The colon length of Group 3 was significantly larger in comparison to Group 1 ($p < 0.001$).

Pathological findings

Macroscopically, nodular and/or polypoid colonic tumors developed in the middle and distal colon of the mice in Groups 1–5. These tumors were histopathologically tubule adenoma (Fig.2*a*) or adenocarcinoma (well and moderately differentiated) (Fig. 2*b*) with a few adenocarcinomas that invaded into the serosa (Fig. 2*c*). A mucosal ulcer (Fig. 3*a*) was also observed surrounding the neoplasms. The enlarged lymph nodes with inflammation were present around the large bowel with tumors. The mice of Groups 6–10 had no tumors in all the organs examined, including the colon. A

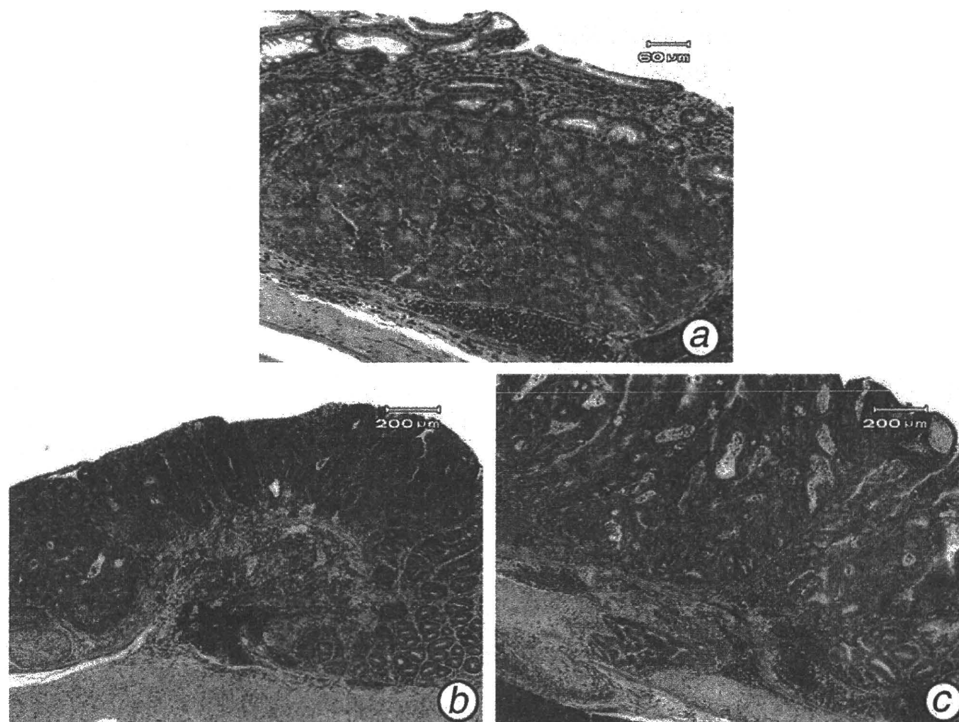

Figure 2. Representative colonic neoplasms induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) in a mouse (Group 1). (*a*) A tubular adenoma, (*b*) a tubular adenocarcinoma with moderately differentiated and (*c*) a tubular adenocarcinoma invaded into the submucosa. Note: The severe inflammation around the tumors; Hematoxylin and eosin stain; the inserted bars indicate magnification (μm).

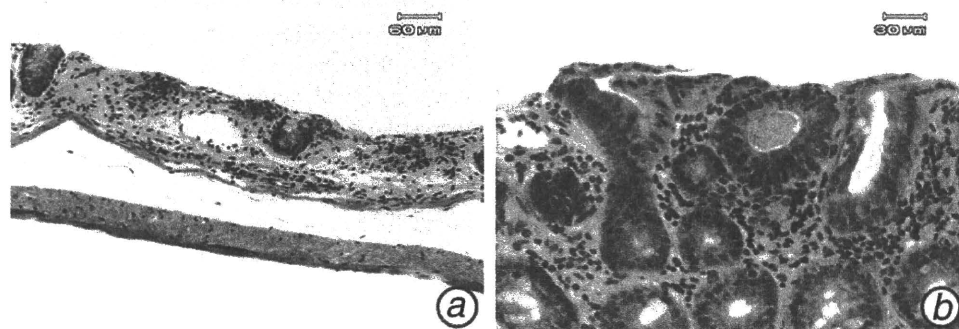

Figure 3. Representative colonic lesions induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) in a mouse (Group 1). (*a*) Mucosal ulcer and (*b*) dysplastic crypts (circled). Hematoxylin and eosin stain, the inserted bars indicate magnification (μm).

mucosal ulcer was observed in the colon of some of the mice of Group 7.

The incidences and multiplicities of the colon neoplasms are summarized in Table 1. Group 1 (AOM/DSS) had 64% incidence of colonic adenocarcinoma with a multiplicity of 1.96 ± 2.24. The incidences of colonic adenocarcinoma of Groups 2 (AOM/DSS → 100 ppm GOFA/β-CD, 24%), 3 (AOM/DSS → 500 ppm GOFA/β-CD, 13%) and 5 (AOM/DSS → 500 ppm AUR/β-CD, 25%) were significantly smaller in comparison to Group 1 ($p < 0.005$, $p = 0.0001$ and $p < 0.005$, respectively). Also, the multiplicities of colonic adenocarcinoma of Groups 2 (0.52 ± 1.16, $p < 0.01$), 3 (0.25 ± 0.74, $p < 0.001$) and 5 (0.42 ± 0.83, $p < 0.05$) were significantly smaller in comparison to Group 1. The incidence (46%) and multiplicity (1.21 ± 1.61) of Group 4 (AOM/DSS → 100 ppm AUR/β-CD) were lower in comparison to Group 1, but the differences between the groups were insignificant. The incidences and multiplicities of colonic adenomas and total colonic tumors in Groups 2–5 were also lower in comparison to Group 1 (Table 1).