Hydrophobic bile acids, genomic instability, Darwinian selection, and colon carcinogenesis

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Abstract: Sporadic colon cancer is caused predominantly by dietary factors. We have selected bile acids as a focus of this review since high levels of hydrophobic bile acids accompany a Western-style diet, and play a key role in colon carcinogenesis. We describe how bile acid-induced stresses cause cell death in susceptible cells, contribute to genomic instability in surviving cells, impose Darwinian selection on survivors and enhance initiation and progression to colon cancer. The most likely major mechanisms by which hydrophobic bile acids induce stresses on cells (DNA damage, endoplasmic reticulum stress, mitochondrial damage) are described. Persistent exposure of colon epithelial cells to hydrophobic bile acids can result in the activation of pro-survival stress-response pathways, and the modulation of numerous genes/proteins associated with chromosome maintenance and mitosis. The multiple mechanisms by which hydrophobic bile acids contribute to genomic instability are discussed, and include oxidative DNA damage, p53 and other mutations, micronuclei formation and aneuploidy. Since bile acids and oxidative stress decrease DNA repair proteins, an increase in DNA damage and increased genomic instability through this mechanism is also described. This review provides a mechanistic explanation for the important link between a Western-style diet and associated increased levels of colon cancer.

Keywords: bile acids, genomic instability, colon cancer

Introduction

Sporadic colon cancer is caused predominantly by dietary factors, most notably a high-fat diet accompanied by low intake of micronutrients (folate, niacin, zinc), antioxidants and other plant phytochemicals. A high intake of fat induces the release of bile acids from the gall bladder into the small intestine. Bacteria in the colonic lumen then convert conjugated and hydrophilic bile acids to unconjugated, hydrophobic bile acids such as deoxycholic acid and lithocholic acid (Figure 1). These hydrophobic bile acids, deoxycholic acid in particular, have been reported to be multiple stress inducers and at high physiologic levels can create chaos within colon epithelial cells. These stresses include membrane perturbation, oxidative DNA damage, decrease in DNA repair proteins, mitotic stress, micronuclei formation, mitochondrial damage (metabolic stress) and endoplasmic reticulum (ER) stress (Figure 2).

Although excessive stress will lead to apoptosis, this altruistic act of removing DNA-damaged cells for the survival of the organism becomes largely circumvented in cells with activated survival pathways. In this "war zone",[1] stimulated by the presence of hydrophobic bile acids, mutated epithelial cells may act in a selfish manner,[2] like bacterial cells, and Darwinian selection[3–10] can come into play at the cellular level. The very stress-response pathways that result in cell survival (eg, activation of nuclear factor-kappa B [NF-κB], autophagy) (Figure 3) can become constitutively upregulated through clonal selection of mutated cells, and can serve to propagate cells even with unrepaired DNA damages and genomic instability. Although it has been known for decades (using animal

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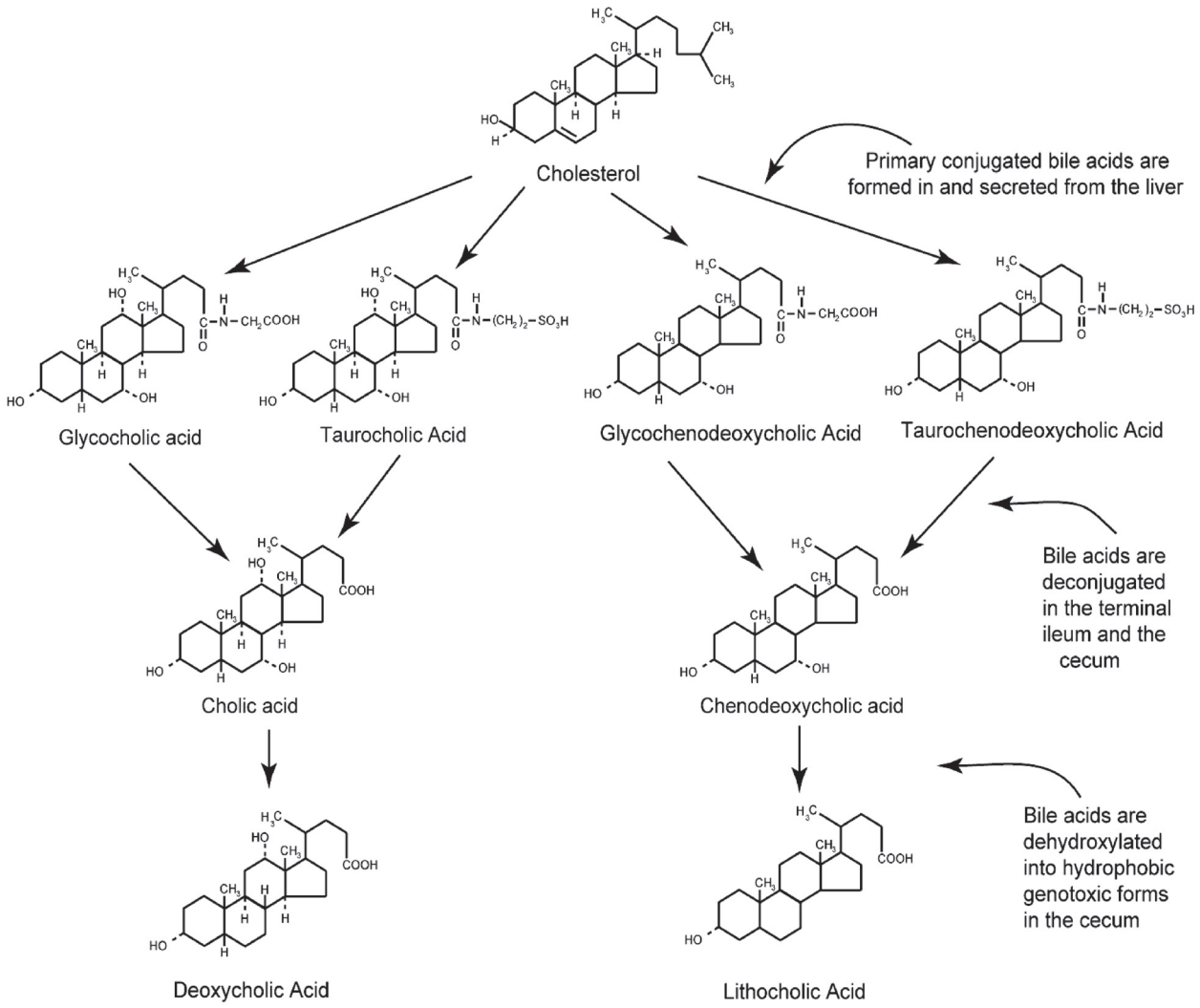

Figure 1 The primary bile acids, cholic acid and chenodeoxycholic acid, are formed in the liver from cholesterol and conjugated, usually with glycine or taurine. They are then secreted into the gall bladder. From the gall bladder, glyco-and tauro- conjugated primary bile acids are released into the duodenum to aid in the digestion of fats. In the terminal ileum and in the cecum, the primary bile acids are deconjugated. Further, in the cecum, they are dehydroxylated through the action of bacterial 7-alpha dehydroxylases. By this reaction, cholic acid and chenodeoxycholic acid are converted to the more genotoxic-hydrophobic bile acids deoxycholic acid and lithocholic acid, respectively.

models of carcinogenesis) that hydrophobic bile acids can promote colon cancer,[11–14] the evidence is now convincing that bile acids are also carcinogens.[15–17] Evidence that bile acids are carcinogens in human gastrointestinal (GI) cancers has been reviewed by Bernstein and colleagues.[15] Proof that hydrophobic bile acids cause mutation in vitro in epithelial cells of the GI tract was provided by the elegant studies of Jenkins and colleagues.[16,17] Even though proliferation is essential to cancer development, it is not the high level of proliferation itself that is responsible for cancer. For example, adenocarcinomas are infrequent in the small intestine, although the proliferation rate of epithelial cells at this site is similar to that of the epithelial cells of the colon. The colon may be particularly susceptible to cancer because it contains tissues that are often exposed to unique stresses (eg, high levels of hydrophobic bile acids resulting from anaerobic bacterial metabolism). Since there may be insufficient time for DNA repair in this "war zone",[18] the selection of mutated cells with a proliferative advantage may occur repeatedly. This proliferative advantage may take the form of apoptosis resistance and/or increased cell division.

How, in particular, do bile acids contribute to colon carcinogenesis? Since somatic mutation rates are normally too low to account for the high incidence of colon cancers, it has been proposed that genomic instability (ie, a mutator phenotype) plays a major role in producing the plethora of random genomic changes needed to produce a malignancy.[19–21] A mutator phenotype can explain the complex histopathology

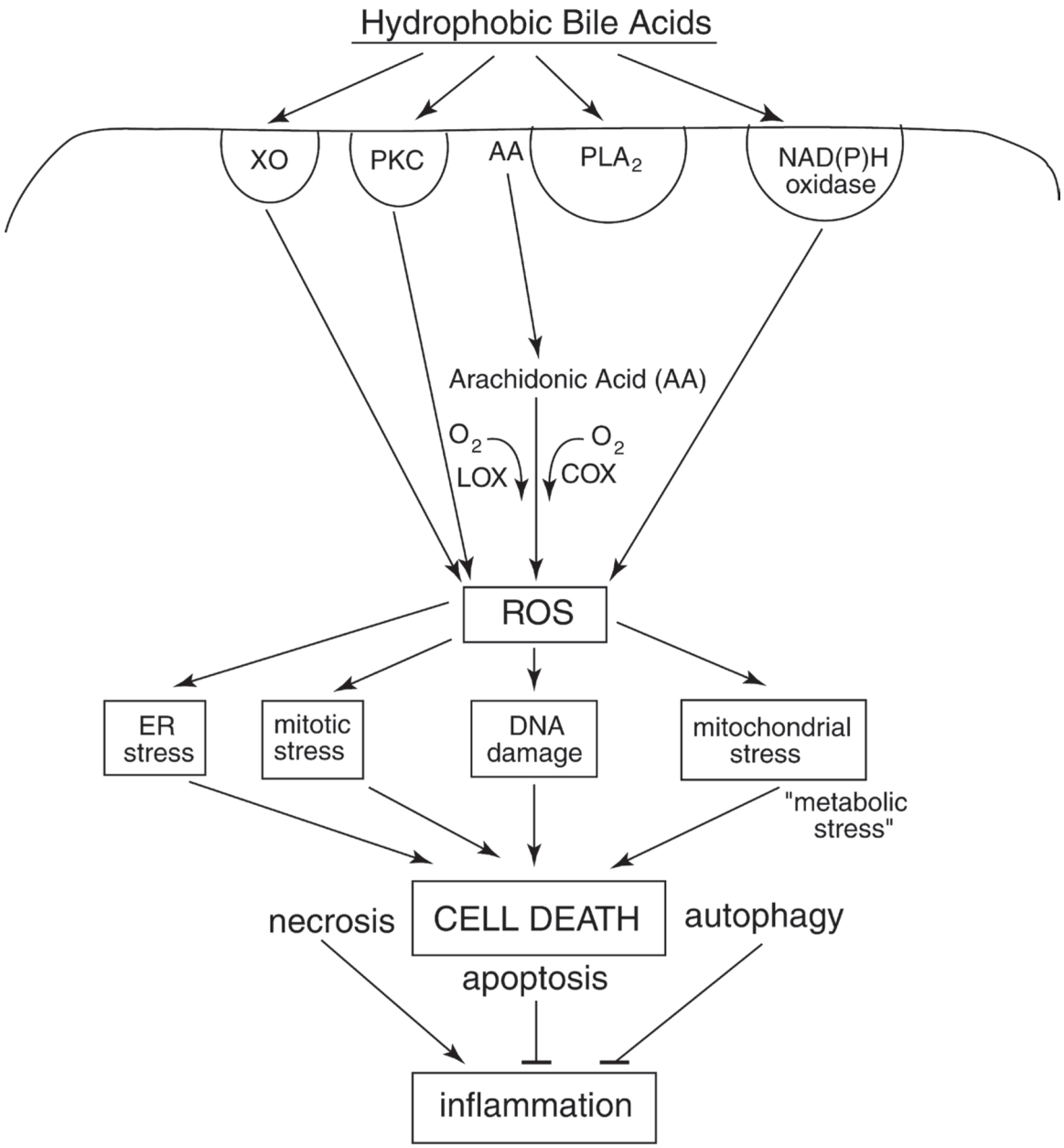

Figure 2 Schematic diagram indicating some of the hydrophobic bile acid (HBA)-induced signaling pathways that begin with the activation of surface enzymes, the subsequent generation of ROS, and the resultant stresses/damages that, if excessive, result in cell death. The most well documented deleterious effects of HBAs in colon cells are DNA damage, mitochondrial stress and ER stress. DNA damage results in the activation of mitotic checkpoint proteins leading to growth arrest. Mitochondrial damage results in the activation of caspases and the cleavage of multiple substrates in the cell. ER stress activates several pro-apoptotic molecules that result in cell death. Repair processes responsive to DNA damage, mitochondrial stress and ER stress can deplete the energy reserves of the cell, resulting in "metabolic stress". Too much stress on the cell results in cell death through mechanisms that involve apoptosis, necrosis, and/or autophagy. HBA-induced mitotic stress can lead to abnormal cell division. Excessive mitotic stress may lead to mitotic catastrophe and cell death, although the details of this mode of cell death have not been well described. Necrotic cells induced by HBA may, especially, elicit an inflammatory response *in vivo,* and this could explain, in part, the induction of colitis by chronic feeding of HBA in mouse models. See text for details.
Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; PKC, protein kinase C; PLA_2, phospholipase A_2; LOX, lipoxygenase; ROS, reactive oxygen species; XO, xanthine oxidase.

and behavior of tumors,[22–24] including different tumor subsets,[25] divergence of clonal populations,[26] and different molecular pathways[27,28] that may be selected during colon cancer progression and lead to different clinical outcomes.[29,30] Genomic instability in cancer appears in three major forms: (i) aneuploidy, in which entire chromosomes are gained or lost, (ii) intrachromosomal instability, characterized by insertions, deletions, translocations, amplifications, and other forms all sharing the feature of utilizing DNA breakage as an early step, and (iii) point or oligobase mutations.[31] Genomic instability is a dynamic process in which the three major forms of genetic instability may co-exist at different stages

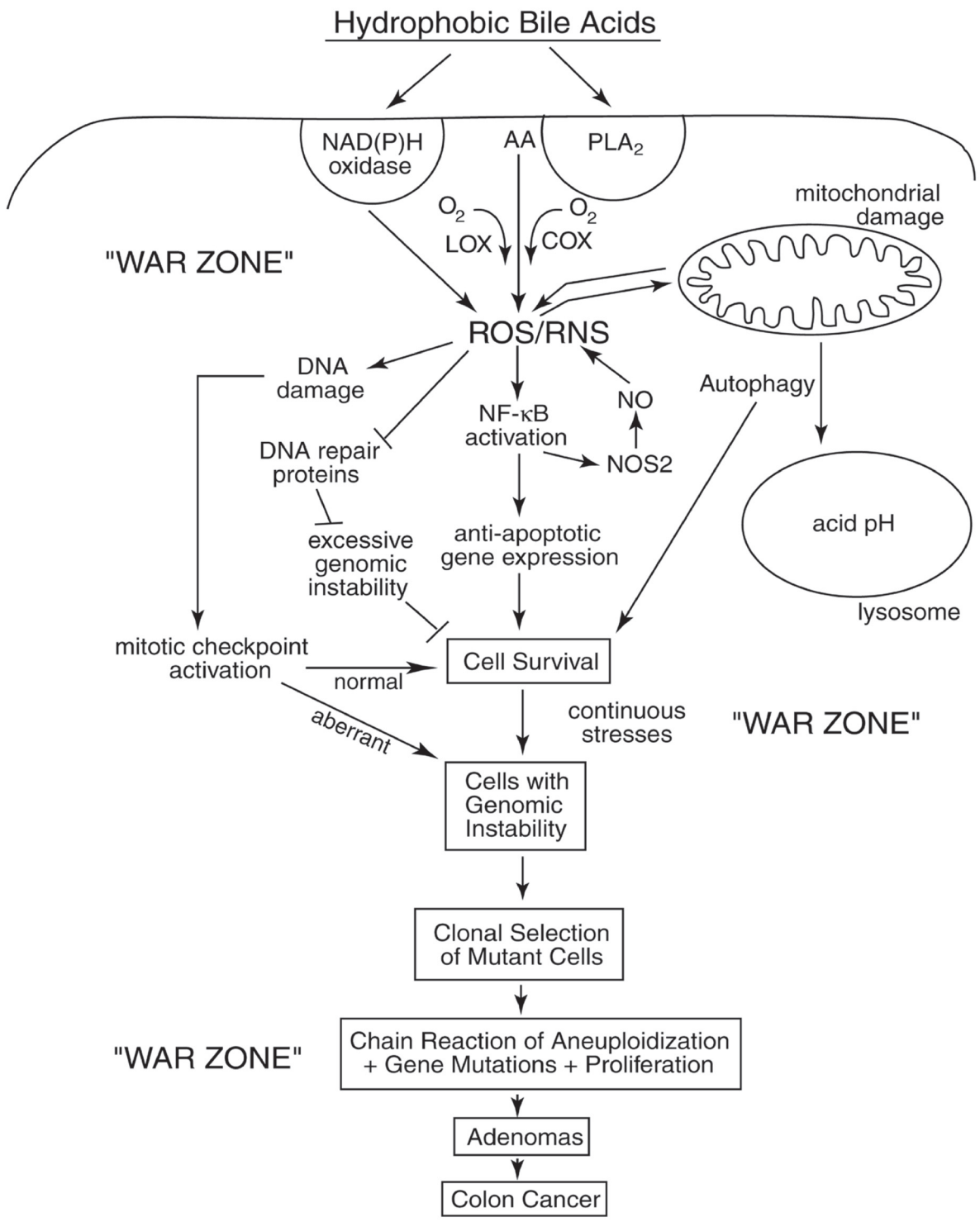

Figure 3 Schematic diagram illustrating a probable sequence of events resulting from exposure to high levels of hydrophobic bile acids (HBAs) that accompany a high-fat diet. This exposure leads to HBA-induced generation of ROS/RNS, activation of survival pathways (eg, autophagy, NF-κB), the generation of cells with genomic instability (eg, mutations, aneuploidy) and clonal selection of mutant cells with survival and proliferative advantages. The end results are the production of adenomas that progress to colon cancer. The epithelial cells of the colon of a person on a Western-style (high-fat/low vegetable/low micronutrient) diet are probably in a persistant "war zone" (bombardment with HBA-induced ROS/RNS, presence of food carcinogens, toxins, etc.). Cells in different stages of progression to malignancy are thus persistently receiving damages to their genome, resulting in clones of cells that are selected for survival in the adverse environment of the colon. While cells in the previous population, if receiving excessive DNA damage, underwent cell death altruistically for the overall benefit of the organism, the new clones of cells may behave selfishly. The new clones may acquire resistance to apoptosis and undergo clonal selection on the basis of their survival advantage, even when their DNA is damaged and after their genomes become unstable. This allows progression to adenomas and colon cancer.

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; LOX, lipoxygenase; PLA_2, phospholipiase A_2; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species.

of colon carcinogenesis, providing support for the chaotic roots of neoplastic development and continuous growth of aberrant clones.[32,33]

Mutations, aneuploidy or other chromosomal aberrations affecting genes that govern genetic stability[5,34–37] could be the cause of a mutator phenotype.[38,39] The occurrence of a mutator phenotype would be made more likely by conditions that cause DNA damage, such as increased oxidative stress, due to persistent exposure to high levels of bile acids that accompanies a high-fat diet. Increased levels of oxidative stress caused by defective mitochondria have been proposed as a possible mechanism by which a "mutator phenotype" could arise.[40] As discussed below, bile acids cause oxidative DNA damage, double-strand DNA breaks (evidenced by activation of phosphorylated H2AX[41]), aneuploidy,[16,17] broken chromosomes[16,17] and aberrant mitoses (unpublished data from our laboratory). In addition, high concentrations of bile acids in the lumen provide a persistent stressful environment that may select for mutated cells that can better survive in that environment (Figure 3). Hydrophobic bile acids thus may serve to both initiate and propagate the mutator phenotype. Cycles of aneuploidization[42] coupled with specific mutations (eg, APC, ras, p53) and large-scale structural alterations to chromosomes (eg, nonreciprocal translocations, inversions, deletions and insertions resulting in "genome scrambling"[43]) may then give rise to a population of cells with multiple karyotypes and genotypes. The clonal selection of cells with particular karyotypes and/or mutations may then produce a "field defect" or "field cancerization" associated with gene expression changes. Frequently, one of the selected genotypes will be apoptosis resistance as found in the normal-appearing flat colonic mucosa of humans[44,45] which is by definition a "survival genotype". Within such a field defect, further mutations may then be selected for increased proliferation, altered cell-cell interactions and/or migratory behavior. On this basis a neoplastic clone can emerge (eg, as found in flat adenomas and polyps). These neoplastic survival karyotypes may undergo further mutation and selection to produce a cancer.

This review emphasizes the plethora of cellular stresses imposed by hydrophobic bile acids. Our review provides possible mechanisms by which a high-fat diet can lead to the development of sporadic colon cancers. The roles of mutations in critical genes (eg, APC, ras, p53) during colon carcinogenesis was previously addressed in the insightful discussions by the Vogelstein group[46–48] and will not be discussed in this review.

Hydrophobic bile acids generate cellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) that damage DNA and proteins. These damages can lead to genomic instability (eg, point mutations and aneuploidy).[49] Point mutations, aneuploidy, and other gross structural alterations of chromosomes (eg, balanced or unbalanced translocations, dicentric chromosomes) appear to interact to produce the unstable and/or aberrant genomes associated with colon carcinogenesis. Aneuploidy is observed in most cancers, although it is not ordinarily found in colonic tumors that have a mismatch repair deficiency and display microsatellite instability.[50] A small subset of colorectal tumors display microsatellite instability, whereas the rest of colorectal tumors display numerical and/or structural chromosomal alterations as the most prominent outcome of genetic disruption.[51]

Loss of heterozygosity (LOH) of a portion of a chromosome or a whole chromosome is already evident at the adenoma stage of colon carcinogenesis[52–65] and is prominent at the colon cancer stage.[59,60,62,63,66–68] A review of the literature indicates that at least 19 of the 22 human somatic chromosomes exhibit aneuploidy during the adenoma to colon cancer stage. The loss or gain of whole or portions of chromosomes involves thousands of genes.[69] This chromosomal imbalance could have dramatic effects on cellular homeostasis, including an increase in oxidative/redox state of the cell and the loss of key DNA repair and pro-apoptotic proteins. Approximately 11,000 genomic events were identified in sporadic colonic polyps using the technique of inter-(simple sequence repeat) polymerase chain reaction (PCR), indicating that genomic destabilization is an early event in sporadic tumor development.[31]

Pre-neoplastic lesions smaller than adenomas are referred to as aberrant crypt foci (ACF).[70,71] The ACF are not identified during routine colonoscopies since vital stains are often not used and biopsies are not usually taken of the flat colonic mucosa. However, human ACF have unequivocally been shown to exhibit genomic instability, including LOH.[72–76] Since numerous genetic alterations and aberrant mitotic changes are also observed in the nonneoplastic colon of mice[77] and humans[78–82] at high risk for colon cancer, biomarkers based on aberrant karyotypes have promise for identifying high-risk individuals. Figures 2 and 3 are based on data from our laboratory and those of others and emphasize the role of hydrophobic bile acids in the induction of multiple stresses and genomic instability. We have selected bile acids as a focus of this review since bile acids play a key role in colon carcinogenesis. We describe how bile acid-induced stresses cause cell death in susceptible cells, contribute to

genomic instability in surviving cells, impose Darwinian selection on survivors in a "war zone" and enhance initiation and progression to colon cancer. This review provides a mechanistic explanation for the important link between a Western-style diet and associated increased levels of colon cancer. However, other factors such as smoking, food-related carcinogens, long-chain fatty acids (eg, palmitic acid), excess iron, arsenic, viruses, bacteria, inflammatory cells, low dietary micronutrients, low omega-3 fatty acids, etc., may also contribute to genomic instability-based carcinogenesis.

Bile acids cause membrane perturbation

It has been known for over thirty years that bile salts perturb plasma membranes, and that deoxycholate is more membrane-damaging than cholate and its conjugates.[83] Binding of bile acids with rat colon and the resultant perturbation of membrane organization was later measured using 31P nuclear resonance spectroscopy.[84] Deoxycholate alters membrane composition, as evidenced by a significant upregulation in membrane cholesterol and phospholipids.[85] Bile acids also induce the modification and upregulation of caveolin-1 in a hydrophobicity-dependent manner, implying widespread receptor dysregulation.[85] These findings were mimicked by other hydrophobic molecules unrelated to bile acids, such as sodium lauryl sarcosine and cholesteryl hemisuccinate, strongly implicating hydrophobicity as an important determinant of bile acid effects on signaling.[85] Deoxycholate also perturbs membrane structure by alterating membrane microdomains.[86] Depletion of membrane cholesterol by treating cells with methyl-β-cyclodextrin suppressed deoxycholate-induced apoptosis, and staining for cholesterol with filipin showed that deoxycholate caused a marked rearrangement of this lipid in the membrane.[86] Additionally, fluorescence anisotropy studies indicated that deoxycholate caused a decrease in membrane fluidity consistent with the increase in membrane cholesterol content measured after deoxycholate treatment.[86]

Bile acids activate surface enzymes, such as phospholipase A_2 (PLA_2),[87,88] epidermal growth factor receptor (EGFR),[86,89–94] Fas receptor,[89] protein kinase C (PKC),[90] bile acid receptor M-BAR/TGR5 (G-protein-coupled receptor),[93] phospholipase C (PLC),[95] nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase[96,97] and the Na^+/K^+ -ATPase.[97] We found that the surface enzymes, Na^+/K^+ -ATPase and NAD(P)H oxidase are involved in activating NF-κB,[97] a redox-sensitive transcription factor upregulated during the development of apoptosis resistance in colon cancer cells,[98] and associated with colon carcinogenesis. The activation of surface and other enzymes through membrane perturbation is, therefore, responsible for modulating signal-transduction pathways in colon cells.[85,86,89,99]

The fact that hydrophobic bile acids cause secretory diarrhea (diarrhea is the most common and debilitating symptom of patients with ulcerative colitis [a pre-neoplastic condition of the colon]), warrants a greater effort to understand how these bile acids induce changes in ion transport.[100] Taurodeoxycholic acid, for example, increases electrogenic Cl^- secretion into the proximal colonic lumen.[101] This stimulated secretion is believed to occur via an increase in intracellular Ca^{++} concentration. Deoxycholic acid reduces transepithelial Na^+ absorption by inhibiting amiloride-sensitive Na^+ channels, and increasing secretion of Na^+, K^+, and Cl^- ions in the distal colon.[102] Hydrophobic bile acids have been reported to act as calcium ionophores,[103,104] most probably by partitioning bile acid/Ca^{++} complexes into the hydrophobic core of the membrane bilayer[104] and/or Ca^{++}-transporting channels.[105] We have recently reported that high cytosolic Ca^{++} levels are a major factor in the activation of NF-κB in HCT-116 colon cells by bile acids.[97] Two agents (EGTA and Ruthenium Red (RuR)) used in that study prevented NF-κB activation by bile acids through at least two different mechanisms. EGTA does not cross cell membranes and chelates Ca^{++} ions in the extracellular milieu, thereby preventing Ca^{++} entry into the cell. RuR is an inorganic polycationic dye that inhibits Ca^{++} influx through voltage-sensitive calcium channels and blocks the release of Ca^{++} from the ER.

Bile acids cause oxidative and nitrosative stress in colon cells

Bile acids induce production of ROS and RNS in colon epithelial cells.[7,106–117] Bile acids generate ROS and RNS by several different pathways, as reviewed by Bernstein and colleagues[15] and summarized here. Bile acids damage mitochondria and damaged mitochondria can "leak" electrons from the electron transport chain to form superoxide (O_2^-). ROS can also be generated by bile acid activation of PLA_2, which releases arachidonic acid from the cell membrane. Arachidonic acid may then be acted on by cyclooxygenase and lipooxygenase to release ROS through partial reduction of O_2, during the conversion of arachidonic acid to prostaglandins and leukotrienes.

Deoxycholate activates the redox-sensitive transcription factor NF-κB in colon epithelial cells.[97–99,110,118–124] When NF-κB is activated it can induce an increase in nitric

oxide synthase 2 (NOS2), an enzyme that can generate micromolar quantities of NO. We have found that repeated exposure of HCT-116 colon epithelial cells to increasing concentrations of deoxycholate results in the consistent upregulation of S-nitrosylated proteins. Some of these post-translationally modified proteins were identified by mass fingerprinting analysis and found to include cytoskeletal proteins, metabolic enzymes, signaling proteins, chaperones, redox-related proteins and differentiation-related proteins.[114] We also found that feeding mice deoxycholate can induce inflammation in a mouse model,[115] and that the inflammation is mediated in large part by RNS, since colitis is markedly attenuated in NOS2 knockout mice.[116]

DNA repair proteins can be modulated by oxidative stress and deoxycholate

The promoting and mutagenic effects of bile acids can be explained, in part, by a decrease in DNA repair proteins. Since oxidative stress is known to reduce expression of DNA repair proteins, bile acid-induced decreases in DNA repair proteins may also be through an oxidative mechanism. The cell types and experimental conditions in which oxidative stress decreases DNA repair proteins, and bile acids decrease proteins involved in 6 different DNA repair pathways are described below. Since DNA repair proteins often have multiple functions in the cell in addition to DNA repair, such as participation in apoptosis[125] and regulation of mitosis[126,127] and checkpoint pathways,[128] a deleterious effect of bile acids on DNA repair protein expression can have important and varied implications for inducing genomic instability.

Oxidative stress causes a decrease in DNA repair proteins

Langie and colleagues,[129] working with human pulmonary epithelial cells A549, showed that oxidative stress caused a reduction in mRNA for the nucleotide excision repair (NER) enzyme ERCC1 as well as a reduction in NER capacity that correlated with level of ERCC1 mRNA. Feng and colleagues,[130] using the human colon epithelial cell line HCT-116 and the human hepatic fetal epithelial line CL-48, showed by in vitro DNA repair synthesis and host cell reactivation assays, that ROS also inhibit NER through production of malondialdehyde. This effect is likely mediated by malondialdehye-induced inactivation of DNA repair proteins. Furthermore, as shown in the human erythroleukemia HEL cell line, oxidative stress inactivates the mismatch DNA repair proteins MSH6 and Pms2 (but not MSH2 or MLH1).[131] In HeLa cells and the human Boleth lymphoblastoid cell line, the base excision repair enzyme OGG1 is degraded or reversibly inactivated following oxidative stress.[132,133]

In mitochondria, DNA polymerase γ is responsible for both mtDNA replication and repair. In an SV40-transformed human fibroblast cell line, the functional ability of the catalytic p140 subunit of DNA polymerase γ is reduced by oxidative stress.[134]

Bile acids decrease DNA repair proteins

Short term exposure to deoxycholate decreases the expresson of the DNA repair proteins p53 and BRCA1

The enzyme p53 has multiple roles in the cell. In particular, it has roles in 6 different DNA repair pathways: (1) homologous recombinational repair, (2) nonhomologous end joining, (3) base excision repair, (4) nucleotide excision repair, (5) mismatch repair, and (6) transactivation of expression of O^6-methyl-guanine-DNA-methyl-transferase for direct repair of alkyl adducts at the O^6-methyl-guanine position.[125,135] While treatment of colon HCT-116 cells with non-cytotoxic (200 μM) deoxycholate concentrations increases mRNA expression of the p53 gene, the level of p53 protein is decreased by 40% through proteasome-mediated degradation.[136] Although mutations in p53 are a late event in colon carcinogenesis, the persistent decrease in p53 function by deoxycholate may have similar consequences as that of a mutation. Work from our group has also shown that deoxycholate decreases BRCA1 at the protein and mRNA levels.[137] BRCA1 has a key role in recombinational repair of DNA damages, and also regulates the function of the centrosomes which carry out mitotic chromosome segregation through spindle organization.[127] Failure to adequately repair DNA damages can lead to replication errors and thus mutation; and failure of mitotic segregation can lead to aneuploidy. Since BRCA1 expression is also reduced in colon adenocarcinomas,[137] it is possible that the modulation of expression of DNA repair enzymes by dietary-related factors may, in part, be responsible.

Long term exposure of colon cells to deoxycholate selects for decreased protein or mRNA expression of DNA repair proteins

Three HCT-116 colon cell lines were developed in our laboratory for stable resistance to bile acids by repeated

exposure to increasing concentrations of deoxycholate over a period of approximately 40 weeks.[98] These cell lines were found to have constitutively decreased protein expression of the DNA repair enzymes MSH3 (mismatch repair), Ku80 (non-homologous end joining), P36/MAT1 (nucleotide excision repair) and XPA (nucleotide excision repair).[138] These cell lines were also found to have decreased expression at the mRNA level for ATM (homologous recombinational repair), MSH3 (mismatch repair) and XRCC4 (non-homologous end joining).[98]

Exposure of cholangiocytes to glycochenodeoxycholate (GCDA) decreases DNA repair enzymes

Komichi and colleagues[139] exposed immortalized mouse cholangiocytes to 200 μM glycochenodeoxycholate (GCDC) for 4 weeks and then performed a microarray gene expression analysis. mRNA expression of OGG1 and MUTYH was downregulated 0.8-fold after exposure to CGDC compared to control cells.

Bile acids cause DNA damage in colon cells

Bile acids induce DNA damage in cells of the colon.[41,90,99,108,109,115,118,140–143] One type of induced damage in colon cells is oxidative DNA damage,[90,108,115,143] suggesting that oxidative stress is a significant cause of the overall bile acid-induced DNA damage in the colon. Exposure of rat colon *ex vivo* with high physiologic concentrations of bile acids resulted in the activation of poly(ADP-ribose) polymerase (PARP).[99] PARP is activated by DNA strand breaks and attaches a polymer of ADP-ribose units to proteins which modulates their functional activity and assists in the opening up of the chromatin to allow for more efficient DNA repair.[144] It is probable that the increase in DNA strand breaks is mediated, in part, through the bile acid-induced increase in ROS and/or RNS. Treatment of human colonic biopsies *ex vivo* with deoxycholate also resulted in the activation of phosphorylated H2AX,[41] implying the generation of double-strand breaks in DNA.[145] Double-strand breaks can cause chromosome breakage with the generation of micronuclei upon exit from mitosis.[16,17] Increased DNA damage may be caused by the bile acid-induced decrease in DNA repair enzymes (see Bile acids decrease DNA repair proteins, above).

Bile acids cause mutation

The repeated observation that bile acids cause DNA damage in colon cells suggests that bile acids also increase the incidence of mutation, since replication of a damaged DNA template strand frequently results in a replication mistake, and thus a mutation. Bile acids have been shown to induce mutation in esophageal cells in culture and *in vivo* in rat esophageal cells. When cultured esophageal cells were treated with deoxycholate, an increase in the frequency of GC to AT mutations in the p53 gene was observed.[16] In other experiments, Big Blue F1*lacI* transgenic rats were subjected to esophagoduodenostomy, a surgical procedure that increases duodenogastroesophageal reflux.[146] In these surgically altered rats, the frequency of mutant *lacI* cells of the esophageal mucosa was significantly greater than in the control rats that were not subjected to surgery. Thus, components of refluxate, such as bile acids, appear to cause mutation. Forty-six percent of the mutant esophageal cells were altered at CpG dinucleotide sites, and most of these mutations (61%) were C to T or G to A transitions. This pattern of mutation seen in the surgical model approximates that seen in human esophageal adenocarcinoma, suggesting that duodenogastroesophageal reflux is carcinogenic. In surgically treated Big Blue mice (rather than rats) that were altered to increase duodenogastroesophageal reflux, increased mutagenesis was also observed.[147] In another rat model of colon carcinogenesis based on exposure to azoxymethane, deoxycholate increased both the incidence of colon tumors and the incidence of tumors with *K-ras* mutations.[148] This finding suggests that deoxycholate may induce *K-ras* mutations. In a sensitive bacterial flucturation test based on the Ames Salmonella test system, bile acids were also found to be mutagenic.[149]

Hydrophobic bile acids induce aneuploidy and micronuclei formation

The induction of aneuploidy by deoxycholate was first reported 26 years ago by Assinder and Uphall[150] in a heterozygous diploid strain of *Aspergillus nidulans*. The detection of aneuploidy was made possible by the unique orientation of mutations on the chromosomes. Each pair of homologues carried mutations, in repulsion, in at least 2 gene loci, those in linkage groups I, III, IV, and V being on opposite sides of the centromere. This orientation allowed a distinction between cross-over and nondisjunctional segregants (aneuploids). It was suggested that aneuploidy was a result of interference with the normal functioning of the mitotic apparatus through disruption of the nuclear membrane. Ferguson and Parry[151] confirmed that bile acids, including deoxycholate, were potent inducers of aneuploidy using the diploid yeast, *Saccharomyces cerevisiae*. The assay used

to detect mitotic chromosome aneuploidy involved scoring of white cycloheximide resistant monosomic colonies. Albertini and colleagues[152] determined that lithocholic acid induces mitotic chromosome loss in the diploid yeast strain, *S. cerevisia*e D61.M. The assay to detect mitotic chromosome loss involved scoring of segregants expressing three linked recessive markers, two of which were located close to the centromere on opposite arms of chromosome VII.

The evaluation of the induction of aneuploidy by bile salts in eukaryotic cells of higher organisms was neglected for almost 20 years, even though a role of bile acids in colon carcinogenesis was established in animal models in the 1970s. The first study of bile acid-induced aneuploidy in cells of multicellular organisms *in vitro* was reported by Jenkins and colleagues[16] using human esophageal cell lines. In this elegant study, the frequency of deoxycholate-induced kinetocore-positive and -negative micronuclei was assessed as a measure of the level of aneuploidy, using the cytokinesis-block micronucleus assay.[153,154] The rationale for this assay is based on data which indicate that aneuploid cells and cells with broken chromosomes will often produce micronuclei after exit from mitosis. The presence of kinetochore-negative micronuclei indicated that chromosome fragmentation occurred, probably as a result of deoxycholate-induced double strand breaks. The deoxycholate-induced increase in micronuclei was significantly reduced by the antioxidant, vitamin C, indicating the importance of diet in the possible prevention of genotoxicity. Unpublished data from our laboratory also indicate that deoxycholate can induce micronuclei in colon epithelial cells *in vitro* in addition to inducing aberrant mitoses (implying perturbation of the mitotic machinery). These unpublished results are consistent with published data from our laboratory that persistent exposure of cells to deoxycholate *in vitro*[98,138] or *in vivo* in an animal model,[115] overall, results in the modulation of genes/proteins involved in chromosome maintenance and cell cycle progression (see Persistent exposure of colon cells to deoxycholate results in the modulation of expression of chromosomal maintenance/ mitosis-related genes, below). These findings indicate that deoxycholate is a true carcinogen,[15] with important implications for dietary intervention strategies to prevent the initiation and/or progression of GI cancer.

A major stress induced by hydrophobic bile acids is the generation of ROS/RNS. Chronic exposure of cells to oxidative stress has been reported to result in increased genomic instability.[155,156] An important source of ROS is damaged/mutated mitochondria.[40,157] The mechanisms by which oxidative stress can lead to genomic instability are probably multifactorial, and include induction of double-strand breaks (resulting in broken chromosomes) and overriding the spindle checkpoint during cell cycle progression,[158] which can result in aneuploidy.

Persistent exposure of colon cells to deoxycholate results in the modulation of expression of chromosomal maintenance/ mitosis-related genes

The induction of aneuploidy with the formation of micronuclei after cells exit mitosis may be caused by a number of defects during cell division. Defects such as insufficient or excessive sister chromatid cohesion,[159,160] kinetochore-microtubule attachment defects,[161,162] failure of DNA damage checkpoints,[163] failure of spindle checkpoints,[164–166] telomere defects[167,168] and aberrant centrosomal organization and multiplicity[169,170] may result in an incorrect number of chromosomes in daughter cells after mitosis. These defects coupled with chromosome breaks can produce an aberrant karyotype that may be carcinogenic. We, therefore, searched for the aberrant expression of mitosis-related genes in colon epithelial cells and colonic tissues that were modulated by deoxycholate. cDNA microarray (Table 1) and proteomic analyses (Table 2) of HCT-116 cell lines persistently exposed to increasing concentrations of deoxycholate *in vitro*[98,138] and cDNA microarray analysis of colonic tissue of wild-type mice receiving dietary supplementation with deoxycholate *in vivo*[115] (Table 3) indicate that deoxycholate modulates the expression of numerous genes associated with mitosis and chromosome maintenance. The list includes 71 genes/proteins associated with cohesion and segregation of chromosomes, telomeres, kinetochore structure, spindle assemby and function, centrosome regulation, various aspects of the cell cycle (metaphase, anaphase), cell cycle progression (kinases, phosphatases, ubiquitin-conjugating enzymes), cell cycle checkpoints and cytokinesis (Tables 1–3).

Since the microarray study was published six years ago,[98] recently identified gene sequences related to mitosis and chromosome maintenance have been added to Table 1 to bring the 2002 study up to date.

In summary, persistent upregulation and/or downregulation of key proteins of the division process by deoxycholate, coupled with decreased DNA repair and increased DNA damage, may lead to genomic instability, including mutator phenotypes. Recent studies have indicated that 1) the spindle proteins, Aurora A and BUB1B, are aberrantly expressed in

Table 1 Fold reduction/induction of mRNA levels of chromosomal maintenance/mitosis related genes in HCT-116 colon cancer cells persistently exposed to deoxycholate

Systematic gene notation	Fold reduction/ induction	Gene and function
AA479781	0.32	Radixin (highly concentrated in the cleavage furrow during the late stages of mitosis)
H24707	0.35	DLG1 (Discs, large (Drosophila) homolog 1 found in the midzone during mitosis); multidomain scaffolding protein; interacts through its guanylate kinase-like domain with KIF13B (kinesin family member 13B)
N69204	0.36	CSE1L (chromosome segregation 1 (yeast homolog)-like); microtubule-associated protein that functions in the mitotic spindle checkpoint; also associates with chromatin and increases the transcription of select p53 target genes, including the PIG3 gene; Ran-binding protein implicated in the nuclear to cytoplasmic reshuffling of importin α, which is necessary for the nuclear transport of several proliferation activating proteins, transcription factors, oncogene and tumor suppressor genes
H23021	0.50	RBBP6 (retinoblastoma binding protein 6); phosphorylated upon DNA damage; co-localizes with mitotic chromosomes and localizes to nucleoli in interpase cells; binds p53 and Rb1; overexpression restricts mitotic progression at prometaphase and promotes mitotic apoptosis
H84048	0.50	RBL1 (retinoblastoma-like 1 (p107); key regulator of entry into cell division; directly involved in heterochromatin formation by maintaining overall chromatin structure; controls histone H4 'lys-20' trimethylation; interacts with transcription factor E2F-4 and with cyclin E/A-CDK2; inhibits cell cycle progression in response to DNA damage in S phase cells; inhibits G1 to S phase progression by downregulating expression of the F-box protein SKp2; reduced expression in colorectal tumors may indicate a poor prognosis
AA490213	0.51	TOB1 (Transducer of p185ERBB2 1 receptor tyrosine kinase); antiproliferative protein; acts as a transcriptional corepressor and suppresses the promoter activity of the cyclin D1 gene through an interaction with histone deacetylase; mice lacking TOB are predisposed to cancer indicating that TOB is a tumor suppressor
R02820	0.52	PDS5A [regulator of cohesion maintenance, homolog A (*S. cerevisiae*)]; component of the molecular glue along with cohesin that maintains sister chromatid cohesion during S phase and maintains it during metaphase; sumoylated at anaphase to promote chromatid separation
R92435	0.54	NUF2R (NDC80 subunit of the outer kinetochore complex component, homolog); localizes to kinetochores from late prophase to anaphase; the NDC80 complex is required for chromosome segregation, spindle checkpoint activity, kinetochore integrity and the organization of stable microtubule binding sites in the outer plate of the kinetochore
AA428749	0.56	PPP1R2 [protein phosphatase 1, regulatory (inhibitor) subunit 2]; alias: PNUTS; serine/ threonine phosphatase involved in exit from mitosis and targeted to the reforming nuclei in telophase following the assembly of nuclear membranes and enhances chromosome decondensation as cells re-enter interphase
AA448676	0.59	UBE2V2 (Ubiquitin-conjugating enzyme E2 variant 2; facilitates progress through the cell cycle but has no ubiquitin ligase activity on its own); plays a role in error-free DNA repair pathway and contributes to the survival of cells after DNA damage
R66447	0.60	MYCN (V-myc avian myelocytomatosis viral related oncogene, neuroblastoma-derived); involved in centrosome amplification
N54344	0.60	NCAPH (non-SMC condensin I complex, subunit H); regulatory subunit of the condensin complex which is required for the conversion of interphase chromatin into mitotic-like condensed chromosomes; the condensin complex introduces positive supercoils into relaxed DNA in the presence of type I topoisomerases and converts nicked DNA into positive knotted forms in the presence of type II topoisomerases; the condensin I complex is required not only to promote sister chromatid resolution but also to maintain the structural integrity of centromeric heterochromatin during mitosis; in NCAPH-depleted cells the pericentromeric and centromeric heterochromatin cannot withstand the forces exerted by the mitotic spindle and undergoes irreversible distortion; highest expression occurs during the G2 phase of the cell cycle

(*continued*)

Table 1 (*Continued*)

Systematic gene notation	Fold reduction/ induction	Gene and function
AA489007	0.60	Aurora borealis (BORA) (binding partner to Aurora A); in interphase cells, Bora is located in the nucleus, but upon entry into mitosis, bora translocates to the cytoplasm (in a cdc2-dependent manner) where it binds to and activates the protein kinase Aurora A. The activation of Aurora A is necessary for centrosome maturation, spindle assembly, and asymmetric protein localization during mitosis
AA608568	0.61	CCNA2 (Cyclin A2); major regulator of cell cycle progression; its synthesis is required for progression to S phase; regulates nuclear envelope breakdown and the nuclear accumulation of cyclin B1; also synthesized during G2/M transition; associated with cyclin-dependent protein kinases 1 and 2; cyclin A2 levels decrease as a result of p53-dependent G2 arrest
AA262211	0.63	DLG7 discs, large homolog 7; HURP (Hepatoma Up-Regulated Protein); mitotic phosphoprotein mediates Ran-GTP-dependent assembly of the bipolar spindle, allows for efficient kinetochore capture at prometaphase, promotes chromosome congression to the metaphase plate, chromosome alignment at the metaphase plate and proper interkinetochore tension for anaphase initiation during mitosis; binds to microtubules through its N-terminal domain which hyperstabilizes spindle microtubules in the vicinity of chromosomes by forming specialized tubulin configurations to form a sheet that wraps microtubule ends; interacts with CDC2 which localizes to the spindle poles in mitotic cells; co-localizes with CDH1 at sites of cell-cell contact in intestinal epithelial cells; phosphorylated by Aurora A which provides a regulatory mechanism for the control of spindle assembly and function; increased expression in the G2/M phase of the cell cycle
R88741	0.67	RBBP8 (retinoblastoma-binding protein 8); tumor suppressor that interacts with CTBP, with the terminal (BRCT) domain of BRCA1, and with the retinoblastoma protein to regulate the G1/S-phase transition of the cell cycle; confers resistance to double-strand break-inducing agents and is recruited to double strand breaks exclusively in S and G2 cell cycle phases
AA448194	1.25	SMN2 (Survival of motor neuron 2, centromeric); function not known at the present time
T90375	1.26	KIF3B (microtubule plus end-directed kinesin motor family member 3B); forms a heterodimer with KIF3A which interacts with SMC3 subunit of the cohesin complex; involved in tethering the chromosomes to the spindle pole and in chromosome movement through plus end-directed microtubule sliding activity *in vitro*; prevents aneuploidy and abnormal spindle formation
AA598887	1.27	SMC1L1 [SMC1 (structural maintenance of chromosomes 1, yeast)-like 1]; a cohesin subunit that is a central component of the cohesin complex required for the cohesion of sister chromatids after DNA replication; forms a heterodimer with SMC3 in cohesin complexes consisting of CDCA5, RAD21, PDS5A/APRIN and PDS5B/SCC-112; before prophase it is scattered along chromosome arms; during prophase, most of the cohesin complexes dissociate from chromosomes except at centromeres where cohesin complexes remain forming part of the kinetochore; phosphorylation of Ser-957 and Ser-966 activates it and is required for S-phase checkpoint activation; role in spindle pole assembly during mitosis; SMC1L1 localizes to centrosomes throughout the cell cycle where it is involved in the organization of dynamic arrays of microtubules
R74078	1.27	TNKS1BP1 [182 kDa tankyrase 1 binding protein 1 that binds to the ankyrin domain (comprises 24 ankyrin repeats) of TNKS1]; serves as an acceptor of poly(ADP-ribosylation) by tankyrase 1 (telomeric PARP); tankyrase 1-mediated polymerization of poly(ADP-ribose) is required for spindle structure and function; TNKS1BP1 co-localizes with chromosomes during mitosis, is phosphorylated upon DNA damage and binds to cytosolic actin in interphase
N68492	1.29	Anaphase-promoting complex (APC) 1; alias is MCPR (meiotic checkpoint regulator); one of the 12 subunits that make up the APC/C, a 20S ubiquitin-ligase complex that targets proteins for proteasomal destruction, an essential step in chromatid separation that is necessary for the metaphase to anaphase transition; phosphorylation of Ser-355 occurs specifically during mitosis;
T75522	1.32	PPP1R10 (Protein Phosphatase 1, regulatory subunit 10); serine/threonine – protein phosphatase involved in chromosome decondensation in a PP1-dependent manner; alias PNUTS; inhibits PPP1CA and PPP1CC phosphatase activities; phosphorylated on Ser-398 and Thr-400 by PKA within the region necessary for interaction with PPP1CA; found in discrete nucleoplasmic bodies and within nucleoli

(*continued*)

Table 1 (*Continued*)

Systematic gene notation	Fold reduction/ induction	Gene and function
W95346	1.34	SAC3D1 (Saccharomyces cerevisiae 3 domain-containing 1 protein); involved in centrosome duplication and mitotic progression (by similarity); localizes to centrosomes in interphase cells and at spindle poles and mitotic spindles at M phase, similar to α-tubulin; functions in the spindle assembly checkpoint; RNA interference suppression of endogenous SAC3D1 causes defects in centrosome duplication and spindle formation resulting in cells with a single centrosome and downregulated Mad2 expression, generating increased micronuclei; increased expression of SAC3D1 by DNA transfection resulted in cells with multiple centrosomes and deregulated spindle assembly with upregulated Mad2 expression until anaphase, generating polyploid cells
AA486312	1.34	CDK4 (cyclin-dependent kinase 4); forms a stable complex with D-type G1 cyclins involved in regulating G1 to S transition; inhibition of CDK4 results in a mitotic delay associated with elevated Wee1 (mitotic delay results from failure of chromosomes to migrate to the metaphase plate; however, cells eventually exit from mitosis, resulting in an increase in cells with multiple or micronuclei); shows aberrant cytological localization in colorectal epithelia in the usual adenoma carcinoma sequence
H18838	1.34	MAB2111 [Mab-21 (C. elegans)-like 1]; meiotic instability associated with the CAGR1 trinucleotide repeat at 13q13
W02403	1.36	CLSPN (Claspin homolog of Xenopus laevis); required for checkpoint-mediated cell cycle arrest in response to inhibition of DNA relication or to DNA damage; acts as a sensor which monitors the integrity of DNA relication forks; expression peaks at S and G2 phases of the cell cycle; phosphorylated in response to replication stress and this phosphorylation is required for its association with Chk1
R21614	1.36	SUPT5H [Suppressor of Ty (S. cerevisiae) 5 homolog]; DS1F large subunit; human chromatin structural protein that is reversibly phosphorylated in mitosis
H45967	1.37	CDK9 (Cyclin-Dependent Kinase 9); cell division cycle 2-like protein kinase 4 which regulates progression through the cell cycle; serine/threonine protein kinase PITALRE; member of the cyclin-dependent kinase pair (CDK9/cyclin-T) complex, also called positive transcription elongation factor b (TERb), which facilitates the transition from abortive to productive elongation by phosphorylating the C-terminal domain of the large subunit of RNA polymerase II and SUPT5H and RDBP, thereby increasing gene expression; forms a CDK9/cyclin-K complex which has kinase activity toward RNA polymerase II; phosphorylates retinoblastoma protein in vitro; phosphorylates p53 on serine 392 independently of CKII; also phosphorylates p53 on serine residues 33, 315 and 392 in a feedback loop between p53 and CDK9, pinpointing a novel mechanism by which p53 regulates the basal transcriptional machinery; upregulated upon exposure to various stresses
R06313	1.38	CROCCL1 (ciliary rootlet coiled-coiled, rootletin-like 1); a major structural component of the ciliary rootlet; recombinant rootletin forms detergent-insoluble filaments radiating from the centrioles; the homopolymeric rootletin protofilaments bundle into variably shaped thick filaments; interacts with C-Nap1 and may function in centrosome cohesion by acting as a physical linker between the pair of centrioles/basal bodies; ciliary rootlet interacts with kinesin light chains and may provide a scaffold for kinesin-1 vesicular cargos; rootletin is phosphorylated by Nek2 kinase and is displaced from the centrosomes at the onset of mitosis; overexpression of rootletin in cells results in the formation of extensive fibers resulting in multinucleation, micronucleation and irregularity of nuclear shape and size, indicative of defects in chromosome separation
AA479771	1.40	CUL7 (Cullin 7); an E3 ubiquitin ligase and a member of the Cullin Ring Ligase family involved in post-translational modifications that are important in the regulation of cell cycle progression; induced by DNA damage and promotes cell growth by antagonizing p53 function; functions as a novel antiapoptotic oncogene
AA455786	1.40	MCM3 [minichromosome maintenance deficient (S. cerevisiae) 3]; required for cell proliferation; acts as a replication licensing factor that acts in early S phase and allows the DNA to undergo a single round of replication per cell cycle; ATM phosphorylates MCM3 on S535 in response to ionizing radiation; overexpressed in various cancers including those of the colon

(*Continued*)

Table 1 (*Continued*)

Systematic gene notation	Fold reduction/ induction	Gene and function
AA032090	1.41	DDX11 [(DEAD/H (Asp-Glu-Ala-Asp/His)] box polypeptide 11); *S. cerevisiae* CHL1-like DNA helicase involved in proliferation; required for sister chromatid cohesion and maintaining chromosome segregation; functions during S, G2, or M phase of cell cycle and is essential for prevention of aneuploidy
N76587	1.41	CDC42BPB (Cell Division Cycle 42 Binding Protein Kinase beta [DMPK-like (dystrophy myotonic protein kinase)]; serine/threonine protein kinase involved in cytoskeletal organization and biogenesis in cell division by acting as a Cdc42 effector
H77797	1.41	KIF12 (Kinesin Family Member 12) contains one kinesin-motor domain involved in microtubule motor activity during cell division
N91750	1.41	E2F2 (E2F transcription factor 2); transcription activator that binds DNA cooperatively with DP proteins through the E2 recognition site, 5'-TTTC[CG]CGC-3', found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA recognition; phosphorylated by CDK2 and Cyclin A-CDK2 in the S-phase of the cell cycle; component of the DRTF1/E2F transcription factor complex that functions in the control of cell cycle progression from G1 to S phase; the E2F-2 complex binds specifically hypophosphorylated retinoblastoma protein RB1; during the cell cycle, RB1 becomes phosphorylated in mid-to-late G1 phase, detaches from the DRTF1/E2F complex, thereby rendering the E2F transcriptionally active
AA488526	1.41	NOLC1 (Nucleolar and coiled-body phosphoprotein 1); shuttles between nucleus, nucleolus and cytoplasm at telophase; begins to assemble into granular-like pre-nucleolar bodies which are subsequently relocated to nucleoli at early G1 phase
T77840	1.42	DLG5 [Discs, large (*Drosophila*) homolog 5]; peripheral membrane protein involved in the negative regulation of cell proliferation; genetic variation associated with inflammatory bowel disease
R70925	1.44	MKS1 (Meckel syndrome, type 1); FABB proteome-like protein required for primary cilium formation; function in mitosis not known at the present time
T67474	1.45	APC7 (anaphase-promoting complex, subunit 7); one of 12 subunits that comprise the anaphase promoting complex, a ubiquitin ligase that controls progression through mitosis and the G1 phase of the cell cycle; The APC7 protein is located in the nucleus during interphase and the centrosome during metaphase/anaphase; functions with other members of the APC complex to regulate sister chromatid separation by degrading securins and targets cyclin B and other destruction box containing proteins for proteolysis; may recruit Cdh1 into the APC complex
AA489602	1.45	TRAP1 (heat shock protein 75); refolds denatured retinoblastoma protein 1 into its native conformation during mitosis; mitochondrial Hsp90 analog; antagonizes ROS generation; protects mitochondria against damaging stimuli; protects cells from granzyme M-mediated apoptosis
AA026709	1.51	DOCK6 (Dedicator of Cytokinesis 6); a Dock-C subfamily guanine nucleotide exchanger, has dual specificity for Rac1 and Cdc42 and regulates changes in the actin cytoskeleton during cell proliferation
H79234	1.51	ACD [Adrenocortical dysplasia homolog (*Drosophila*)]; telomeric regulator and component of the TRF1 complex controlling telomere length; controls POT1 telomeric recruitment and telomere elongation by inhibition of telomerase activity
AA448755	1.54	CDC25B (cell division cycle 25B); tyrosine phosphatase 2 that acts as a M-phase inducer; directly dephosphorylates CDC2 and stimulates its kinase activity; activates centrosomal Cdk1 in late prophase; activity controlled by centrosome-associated Chk1 which phosphorylates CDC25B in the absence of DNA damage; cooperates with Cdc25A to induce mitosis, but has a unique role in activating cyclin B1-Cdk1 at the centrosome resulting in centrosome separation
R93686	1.61	MPHOSPH9 (M-phase phosphoprotein 9); involved in regulation of progression through the cell cycle
R93719	1.65	GSPT1 (G1 to S Phase Transition 1 protein); GTP-binding protein 1 essential for G1 to S transition of the cell cycle; alias is eRF3 (eukaryotic release factor 3), which is a GTPase associated with eRF1 in a complex that mediates translation termination

(*Continued*)

Table 1 (*Continued*)

Systematic gene notation	Fold reduction/ induction	Gene and function
AA459292	1.67	CKS1B (CDC28 protein kinase regulatory subunit 1B); binds to and activates cyclin-dependent kinases and also interacts with SKP2 to promote the ubiquitination and proteasomal degradation of p27(Kip1); cyclin D1 regulates CKS1B-mediated degradation of p27(Kip1); exhibits altered expression in colorectal carcinoma
AA076063	1.71	CALD1 (Caldesmon 1); actin-binding protein; during mitosis caldesmon dissociates from microfilaments
R40850	1.74	ARP1 [Actin-Related Protein 1, yeast homolog A (centractin alpha)]; ACTR1A; major subunit of dynactin, a multiprotein complex known to contain 8 or 9 Arp1 monomers in a 37 nm filament involved in microtubule-based vesicle motility; attachment site for cargo directed to the dynein/dynactin complex; centrosome-associated actin homolog; overexpression results in cell cycle delay at prometaphase with appearance of supernumerary microtubule asters
AA488221	1.83	DCTN1 [Dynactin 1 (p150, Glued) Drosophila homolog]; largest subunit of the 10 components that make up the large macromolecular dynactin complex that is involved in "search-and-capture" mechanisms that include the attachment of microtubules to kinetochores during mitosis, the maintenance of the spindle, formation of astral microtubules, chromosome motion and chromosome segregation; requires Aurora kinase B activity to be maintained at kinetochores; binds directly to microtubules and to cytoplasmic dynein; co-localizes with Arp1 to spindle microtubules; depletion in cells results in a metaphase delay and the poor connection of the centrosomes to the mitotic spindle poles; p150(Glued) is cleaved during apoptosis
T99336	1.88	CEP164 (centrosomal protein of 164 kDa localized to the outer appendage); mediator protein required for the maintenance of genomic stability through the modulation of MDC1, RPA and CHK1; key player in the DNA damage-activated signaling cascade; phosphorylated upon replication stress; DNA damage-induced phosphorylation of CHK1 and activation of the G2/M checkpoint requires Cep164; plays a role in chromosome segregation, in addition to its function in checkpoint signaling; persists at centrioles throughout mitosis
R31831	2.00	SHROOM3 (F-actin binding protein); induces cell elongation by redistributing γ-tubulin (associated with centrosomes) and directing microtubule arrays

Notes: Fold reduction/induction is the ratio of mRNA levels in the deoxycholate-treated cells to the level in the untreated control cells. All fold reduction/induction ratios are the mean of all resistant cell lines HCT-116RB, HCT-116RC, and HCT-116RD compared to long-passage untreated cells and were statistically significant at the 95% probability level ($p < 0.05$).

dysplastic mucosa of patients with longstanding ulcerative colitis,[171] a pre-neoplastic condition), 2) both Aurora A and BUB1B undergo a shift in subcellular localization during malignant transformation,[172] 3) a reduced level of the spindle checkpoint protein, BUB1B, is associated with aneuploidy in colorectal cancers,[173] and 4) chromatid cohesion defects may underlie chromosome instability in human colorectal cancers.[174] It is an intriguing possibility that these defects occur very early during colon carcinogenesis, and may be caused, in part, by dietary-related factors, such as high levels of endogenous bile acids, coupled with polymorphisms in mitotic checkpoint genes.[175]

Oxidative stress as an important cause of genomic instability

The induction of kinetochore-positive and -negative micronuclei by bile acids may result from aneuploidy (involving the loss and gain of whole chromosomes) or portions of chromosomes, resulting from unrepaired double-strand breaks. This bile acid-induced induction of genomic instability is most probably caused by oxidative stress directed at parts of the mitotic machinery, checkpoint proteins and/or direct oxidative damage to DNA that results in double-strand breaks. The only studies that addressed this issue in the gastrointestinal tract are those of Jenkins and colleagues,[16,17] who evaluated several antioxidants (eg, vitamin C, resveratrol, EGCG) to determine their effectiveness at reducing deoxycholate-induced micronuclei formation in esophageal cells. Of the antioxidants tested, only vitamin C had a significant effect on reducing micronuclei formation. Since bile acids damage mitochondria, resulting in an increase in mitochondrial ROS, and ROS derived from damaged mitochondria are known to induce genomic instability,[157] various antioxidants specifically directed at mitochondria should be tested for their effectiveness at reducing bile acid-induced genomic instability.

Table 2 Statistically significant increases/decreases in protein levels of chromosomal maintenance/mitosis related genes in HCT-116R colon cancer cells persistently exposed to deoxycholate

Change in expression[1] in resistant cell lines			Gene and function
B	**C**	**D**	
↓	↓	↓	KIF1A (Kinesin Family Member 1A); transports membrane-bound organelles toward the plus end of microtubules and important in cell division; functions in monomeric and dimeric states as a kinesin motor
↓	↓	↓	Cyclin A (required during S phase and passage through G2); activates Cdk2 near the start of S phase and is necessary for the initiation of DNA replication; also binds to cdc2 (Cdk1); mutation or disruption of normal Cyclin A in cells results in a G2 arrest
↓	↓	↓	EB1 [strong binding to the C-terminal domain of APC (adenomatous polyposis coli)]; interacts with the plus end of microtubules and targets APC to microtubule tips]; also required for the plus-end localization of CLIP-170 which is then required to localize p150(Glued) to plus-ends; targeted disruption of the interaction between EB1 and p150(Glued) suppresses anaphase astral microtubule elongation and a delay of cytokinesis
↓	O	↓	SGT1 (G1/S and G2/M cell cycle transitions); important component in association with Skp1, Cul-I, F-box protein and CDC34/Ubc3 of the SCF ubiquitination ligase complex responsible for cell cycle transitions
↓	O	↓	Chk1 (checkpoint kinase 1); activated after DNA damage; leads to G2/M arrest; regulates the S phase checkpoint by increasing the proteolysis of Cdc25A; cooperates with p21 to prevent apoptosis during DNA replication fork stress; inhibition causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage
O	↓	↓	MAD2 (mitotic arrest-deficient 2); required for spindle assembly during mitosis and mitotic checkpoint control; localizes to the kinetochore of condensed chromosomes during mitosis; participates in checkpoint inhibition of the APC/C through a complex of BUBR1, BUB3 and CDC20; prevents premature proteolysis of cyclin B and securin; reduced cellular levels result in defective mitotic checkpoint control; averts aneuploidy by delaying anaphase onset until chromosomes align; functions as a tumor suppressor
O	↓	↓	Eg5 (member 11 of the kinesin-5 family of microtubule-based motor proteins involved in mitosis and cytokinesis); phosphorylated exclusively on serine during S phase, but on both serine and Thr-926 during mitosis, thereby controlling the association of Eg5 with the spindle apparatus (probably during early prophase); important for bipolar spindle assembly and spindle function during mitosis; the rate of bipolar spindle assembly depends on the microtubule-gliding velocity of Eg5; Eg5 is also phosphorylated during mitosis at Thr-297, an evolutionarily conserved cdc2 phosphorylation site, by p34[cdc2]/cyclin B; phosphorylation by p34cdc2 regulates binding of Eg5 to the dynactin subunit 150(Glued); inhibition of phosphorylation blocks the interaction of Eg5 with centrosomes arresting cells in mitosis with monoastral microtubule arrays
O	↓	↓	P140mDia (protein of 140mkDa); mammalian homolog of Drosophila's diaphanous essential for cytokinesis
↑	↓	↑	PTP1D/SHP2 (cytosolic Protein Tyrosine Phosphatase 1D/Src homology-2 (SH2) domain-containing phosphatase 2); helps maintain intracellular protein phosphotyrosine homeostasis and cell cycle progression
↑	↑	↑	hRad9 (phosphorylated by ATM in response to DNA damage); checkpoint control; required for phosphorylation of Chk1; plays a role in cell cycle arrest during the G2 phase before entry into mitosis; phosphorylated hRad9 is found in the nucleus after DNA damage, and forms DNA damage-responsive complexes with other putative checkpoint control proteins, such as hRAD1 and hHUS1; binds the anti-apoptotic proteins Bcl-2 and Bcl-xL
↑	↑	↑	Ran (Ras-related nuclear protein); highly conserved GTPase implicated in DNA replication and entry and exit from mitosis; at steady-state, 80%–90% of cellular Ran is located in the nucleus, with the remainder in the cytoplasm

(*Continued*)

Table 2 (*Continued*)

Change in expression in resistant cell lines			Gene and function
B	**C**	**D**	
O	↑	↑	Cyclin D3 (regulatory subunit for cyclin-dependent kinase Cdk5); interacts with Cdk4 which is required for G2 phase cell cycle progression; regulator of progression through G1 phase during the cell cycle; promoter of cyclin D3 is regulated by E2F1; interacts with p58(PITSLRE), a G2/M-specific protein kinase; overexpression is associated with the accumulation of p27(kip1); interaction partner of lamin A/C; downregulated by rapamycin; when bound to cdks, the D-type cyclins also associate with the polymerase-delta subunit, PCNA; also binds pRb *in vitro*, suggesting that pRb may be an *in vivo* substrate of D-type cyclins; activates caspase 2, connecting cell proliferation with cell death; involved in liver metastasis of colorectal cancer
O	↑	↑	Lap2 (Lamina-associated polypeptide 2); integral protein of the inner nuclear membrane; binding to lamins contributes to the attachment of the nuclear lamina to the inner nuclear membrane; organization of the nuclear envelope during cell cycle progression; also binds to chromatin, implying its role in chromosomal organization during mitosis; mitotic phosphorylation of LAP2 regulates its binding to lamins and chromosomes during the disassembly and reassembly of mitosis

Notes: Increase (↑), Decrease (↓), No Change (O).

Since this area of bile acid research is just beginning to be explored, we have reviewed the literature involving other cell types (beyond those of the GI tract) and found that oxidative stress plays a definite role in the induction of genomic instability.[40,156,157,176–178] Aneuploidy may often reflect defects in mitotic segregation in cancer cells.[50] The faithful cohesion and separation of chromosomes are controlled by a large number of proteins, some of which have a checkpoint function.[180–185] Increased production of ROS has been reported to induce aneuploidy by impairing the spindle check-point function.[158] The spindle checkpoint is the safeguard mechanism that halts anaphase onset until the mitotic spindle has been assembled and metaphase chromosomes properly attached. At the metaphase-anaphase transition, the APC (anaphase-promoting complex) initiates the separation of sister chromatids by catalyzing the ubiquitination of securin, a protein that prevents the proteolysis of the cohesin complex and the subsequent segregation of the chromosomes, in the securin-separase complex.[186] The APC11 subunit functions as the catalytic core of the APC complex and contains a RING-H2-finger domain, which includes one histidine and seven cysteines residues that coordinate two Zn^{2+} ions. The RING-H2-finger domain is a target of hydrogen peroxide, which induces the release of of bound zinc as a result of the oxidation of cysteine residues.[187] The oxidation of APC11 reduces the normal functions of the unoxidized APC11 protein, resulting in the inhibition of ubiquitination and degradation of cyclin B1, and the degradation of securin. Other chromosomal aberrations, such as translocations and dicentric chromosomes, may be caused by accumulation of double-strand breaks induced by oxidative damage, as in Werner syndrome cells.[188] Based on the data from our laboratory that shows the modulation of 71 genes involved in chromosome maintenance, chromatid cohesion, kinetochore-microtubule attachments, chromatid separation, cytokinesis, etc. by deoxycholate (see Tables 1–3), some of these proteins may be oxidatively damaged and/or targeted for destruction by deoxycholate. However, no studies have yet addressed this important mechanism of bile acid-induced damage to dividing cells. Thus, in colon carcinogenesis, the persistent increase in oxidative stress in epithelial cells caused by excessive exposure to bile acids may induce mitotic defects very early in the sequence of events that lead to colon cancer. Hydrophobic bile acids damage mitochondria, and increased levels of oxidative stress caused by defective mitochondria have been proposed as a possible mechanism by which a "mutator phenotype" could arise.[40]

Bile acids induce ER stress

The induction of ER stress by hydrophobic bile acids is evidenced by an increase in Gadd153/CHOP,[142,189,190] a pro-apoptotic transcription factor,[191–197] and an increase in GRP78,[98,190] a molecular chaperone that protects against ER stress.[197,198] Furthermore, hydrophobic bile acids dilate the cisternae of the endoplasmic reticulum (identified using transmission electron microscopy[199]). The mechanisms by which bile acids cause ER stress probably include the generation of ROS that may damage the ER membrane and/or cause protein unfolding, and the release of Ca^{++} from the ER through the activation of inositol triphosphate and ryanodine receptors.[200] Excessive ER stress may lead to cell death in the form of apoptosis

Table 3 Fold reduction/induction of mRNA Levels of mitosis-related genes in colon of mice fed a diet supplemented with deoxycholate

Fold reduction/induction	Gene and function
0.50	Wee1 homolog (*Schizosaccharomyces pombe*); protein kinase that inhibits Cdc2 activity, thereby preventing cells from proceeding through mitosis; causes G2 arrest
0.55	APC (adenomatous polyposis coli); tumor suppressor protein that regulates free β-catenin levels and participates in Wnt signaling; binds to microtubules and increases microtubule ability; APC binds to microtubule plus ends and promotes microtubule net growth with or without EB1; haploinsufficiency accounts for a fraction of FAP patients without APC truncating mutations; decrease in APC expression after one week of supplemental deoxycholate feeding is dependent on the presence of NOS2 (inducible nitric oxide synthase) (unpublished data from the Bernstein et al 2006 study[115])
1.24	CDC2L5 (cell division cycle 2-like 5); serine/threonine kinase; controller of the mitotic cell cycle;
1.32	Ubiquitin protein ligase E3C; E3 ubiquitin ligases degrade proteins via the ubiquitin-proteasomal pathway during mitosis
1.33	Cdc34 (cell division cycle 34); homolog of *S. cerevisiae*; Ubc3 ubiquitin-conjugating enzyme that controls proliferation through the regulation of p27 kip1 protein levels; mediates the degradation of wee1; inhibits the association of CENP-E with kinetochores and blocks the metaphase alignment of chromosomes
1.34	Tsg101 (Tumor susceptibility gene 101); necessary for cell proliferation and cell survival; may function as a dominant-negative inhibitor of ubiquination in pathways where protein expression is tightly regulated; interferes with MDM2 ubiquination leading to a decrease in MDM2 decay and downregulation of p53 protein
2.10	CDK5 (cell division protein kinase 5); involved in control of G1 to S phase transition of the cell cycle; one of the two main tau-kinases, complexing with cyclin D (D1, D2, D3); hyperphosphorylation of tau reduces tau's ability to associate with microtubules; phosphorylates the tubulin-binding protein, stathmin; phosphorylates p53 and regulates its activity

and/or necrosis. Since deoxycholate induces the formation of nitrotyrosine residues in proteins of colon cells,[112] ER stress may also be mediated through the generation of RNS.[195,201] Persistent ER stress may then select for cells that exhibit activated ER stress-responsive survival pathways important in colon carcinogenesis. We have shown that ER stress-related survival proteins are consitutively upregulated in colon cancer cells that are resistant to deoxycholate-induced apoptosis, and include Grp78,[98] S-nitrosylated Grp78[114] and S-nitrosylated ORP150,[114] another inducible ER stress-related chaperone protein.[202] The observed constitutive increase in autophagic proteins[98] observed in these resistant cells may also serve to protect against ER stress, although this area of research has not been fully explored.

Consequences of bile acid-induced mitochondrial damage: metabolic stress, autophagy, apoptosis, and necrosis

Bile acid-induced DNA damage, ER stress, mitotic stress, and mitochondrial damage may all lead to cell death through various processes including apoptosis, necrosis, autophagic cell death and/or mitotic catastrophe, depending on the level of combined stresses. Of all organelles, mitochondria are most central to the three main cell death pathways, apoptosis, necrosis, and autophagic cell death.[203] Apoptosis as a mechanism of mitochondrially-mediated bile acid-induced cell death has been studied the most in cells of the GI tract, as discussed below.

Mitochondria generate most of the ATP used for energy-consuming processes within the cell. Moreover, mitochondria play an important role in the regulation of other cellular processes such as autophagy and apoptosis. Autophagy is a catabolic process involving the degradation of a cell's own components through the activation of lysosomes. In response to stresses induced by hydrophobic bile acids, autophagy is initiated (unpublished data from our laboratory) as the first mechanism to cope with damage when mitochondrial injury is limited. The damaged mitochondria are sequestered within lysosomes, thereby preventing the release of apoptosis-inducing factors, such as cytochrome c, apoptosis-inducing factor (AIF), and Smac/DIABLO. As more mitochondria are damaged and undergo the mitochondrial membrane permeability transition in response to bile acids, the pro-apoptotic

proteins are released from the mitochondria.[204] These releases result in the formation of the apoptosome, caspase activation, degradation of vital proteins in the cell and, finally, apoptosis. Apoptosis can be triggered by two distinct signaling cascades, the extrinsic and intrinsic pathways. The extrinsic pathway involves the activation of specific cell surface receptors such as Fas/CD95 and the tumor necrosis factor receptor (TNFR), while the intrinsic pathway is primarily regulated by mitochondria. Importantly, in contrast to hepatocytes and hepatic cancer cell lines, in which hydrophobic bile acids induce apoptosis by an extrinsic pathway, deoxycholate induces apoptosis in colonic epithelial cells in a CD95-independent pathway.[205] Bile acid-induced mitochondrial damage may initially increase autophagy to rid cells of damaged organelles. As stress continues, apoptosis or autophagic cell death may follow. If, however, the stresses on mitochondria are initially severe, oxidative phosphorylation fails, NAD^+ levels drop, ATP levels are rapidly depleted, resulting in loss of ionic control,[206] followed by cell swelling and lysis, characteristic features of necrosis.[207–211] Apoptotic cell death induced by deoxycholate has been frequently reported in colon epithelial cells.[41,44,45,98,99,110,112,118,199,205,212–224] Therefore, an understanding of bile acid-induced alterations in mitochondrial function are of great interest. At least two major alterations occur during bile acid-induced mitochondrial dysfunction. These are (1) disruption of electron transport leading to generation of ROS, loss of mitochondrial membrane potential (MMP), decrease in oxidative phosphorylation, and decrease in ATP production, and (2) release of mitochondrial proteins that trigger activation of caspases. Bile acids were shown to be involved in all three processes.

The exact mechanism by which bile acids disrupt electron transport and decrease oxidative phosphorylation has yet to be defined.[225–227] Experiments performed with mitochondria isolated from hepatocytes clearly demonstrate that hydrophobic bile acids induced perturbations in mitochondrial bioenergetics.[225,226] Specifically, complex I and complex III of the mitochondrial electron transport chain were inhibited by treatment with10 μM bile acid. Complex IV was inhibited at higher concentrations of bile acids.[225] The authors speculated that the inhibition of the electron transport complexes may be due to bile acid incorporation into the mitochondrial membranes resulting either in direct toxicity or alteration in lipid composition. When they tested this hypothesis, they found that, indeed, bile acids were incorporated into the mitochondrial membranes. Furthermore, they found that the hydrophobic bile acid, chenodeoxycholic acid, induces alteration in the lipid composition of the mitochondrial membrane, while no alterations were found after treatment with lithocholic acid.[225] However, it is possible that the changes in lipid composition induced by lithocholic acid were below the limit of detection used in the study.[225] Membrane structural changes observed using spin-labelling techniques and electron paramagnetic resonance (EPR) spectroscopy analysis also support the involvement of mitochondria in bile acid-induced apoptosis.[228] These mitochondrial structural changes include modified lipid polarity and fluidity, altered protein order and increased oxidative injury.[228]

Hydrophobic bile acids cause mitochondrial oxidative stress[97,110,117,228,229] leading to mitochondrial swelling[199] and formation of megamitochondria.[110] The excessive generation of ROS that overwhelms the cells' antioxidant defenses is most probably responsible for the death of colonic epithelial cells,[230] and is consistent with the role of oxidative stress in mediating apoptosis in many other types of cells.[231] Importantly, bile acid-induced apoptosis in HT-29 colon cells is dramatically reduced if mitochondria are protected against ROS by inhibiting complexes I and II of the electron transport chain.[110] Later studies indicated, moreover, that perturbation of the mitochondrial electron transport chain, in general, and uncoupling of oxidative phosphorylation, attenuates deoxycholate-induced apoptosis.[199] The mechanism by which this perturbation of mitochondrial function results in apoptotis resistance is not clearly understood. Another effect of hydrophobic bile acids on mitochondria is rapid loss of mitochondrial membrane potential (MMP).[110,199,205] For example, treatment of SW480 cancer cell line with 0.5 mM deoxycholic acid induced loss of MMP in 5 minutes.[205] This loss of MMP correlates with the induction of ROS.

The second major effect of hydrophobic bile acids is to induce rapid release of pro-apoptotic proteins in colon cancer cells, such as cytochrome c, which leads to the activation of caspases.[218] Incubation of HT-29 and HCT-116 colon cells with deoxycholate resulted in the cleavage of procaspases and the activation of effector caspases 2, 3, 7 and 8,[110,222] while caspase-1 proinflammatory caspase was not cleaved.[222] Caspases 9 and 3 are rapidly activated by deoxycholate, while caspase 8 activation is a later event.[110,218,222] Caspase-6 is of particular importance to deoxycholate-induced apoptosis of colon epithelial cells, since the antiapoptotic nitric oxide-targeted enzyme, guanylate cyclase-α1, is specifically cleaved by caspase-6, resulting in apoptosis.[224] Apoptosis in HCT116 cells is not induced simply by a detergent effect, since sodium dodecyl sulfate (SDS), a detergent that is structurally distinct from bile acids, does not induce apoptosis.[218] Although protein synthesis inhibitors have been shown to

protect against programmed cell death, *de novo* synthesis of proteins does not appear to be necessary for bile acid-induced apoptosis.[218] Altogether, these data suggest that in contrast to the extrinsic induction of apoptosis, as seen in hepatocytes, hydrophobic bile acids primarily activate the intrinsic mitochondrial pathway of apoptosis in colon cancer cell lines.

How exactly do bile acids interact with mitochondria to perturb their function? Hydrophobic bile acids may directly affect mitochondria, leading to disruption of the electron transport chain and increased production of mitochondrial ROS. Alternatively, mitochondria can be damaged indirectly by extramitochondrial ROS produced by plasma membrane bound enzymes targeted by bile acids, such as NAD(P)H oxidase[96] (Figure 2). The importance of NAD(P)H to deoxycholate-induced apoptosis was ascertained by the use of an inhibitor of NAD(P)H oxidase, diphenyleneiodonium (DPI).[232] DPI attenuated overall ROS production and prevented the occurrence of deoxycholate-induced apoptosis, assessed using translocated membrane phospholipid and nuclei containing condensed chromatin.[232] Interestingly, NADPH oxidase levels are elevated in colon cancer cell lines as well as in the tissues from patients with colon cancer.[233–236] Another extramitochondrial source of ROS occurs when molecular oxygen is partially reduced during the metabolism of arachidonic acid by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Figure 2). Another indirect effect of bile acids on mitochondria is the release of arachidonic acid from phospholipids following activation of PLA_2 by bile acids.[87] Arachidonic acid induces inhibition of mitochondrial complex I and III and causes an increase in mitochondrial ROS production.[237] Hydrophobic bile acids may also trigger the formation of ceramide,[238] which can damage mitochondria and induce apoptosis. The ceramide-induced disruption of mitochondrial function and induction of apoptosis is caused, in part, by modulation of gene expression of members of the Bcl-2 family of apoptosis-related proteins.[239]

Persistent exposure to deoxycholate selects for apoptosis resistance

Repeated long-term exposure of colon epithelial cells to high physiologic concentrations of bile acids may allow preferential survival of cells that are resistant to induction of apoptosis by bile acids. Such apoptosis resistant cells could arise and proliferate by the processes of mutation and Darwinian selection (Figure 3). Apoptosis-resistance in the colon appears to arise in settings where the colonic mucosa is chronically exposed to high physiologic levels of bile acids. Magnuson and colleagues[240] reported that chronic feeding of 0.2% cholic acid (added to the AIN-76 diet) to azoxymethane (AOM)-treated Sprague-Dawley rats for 18 weeks resulted in a statistically significant reduction in the number of apoptotic bodies in ACF after an acute low dose of AOM, compared with normal-appearing crypts. Both normal and ACF crypts had fewer apoptotic bodies per 100 cells than crypts from rats fed the control diet.

Work from our laboratory indicated that *in vitro* exposure of apoptosis-sensitive colon epithelial cells (HCT-116) for approximately 40 weeks resulted in the generation of stable apoptosis-resistant cell populations.[98] We also evaluated the nonneoplastic flat colonic mucosa of patients in different colon cancer risk groups for apoptosis competence using an ex vivo live cell bioassay.[44,45,212,213,241] The apoptosis-inducing agent used in this bioassay was deoxycholate. The nonneoplastic colonic mucosa of patients with colon cancer had a significantly reduced apoptotic index compared to that of low-risk and normal patients. The exact etiologic agents that were responsible for the development of apoptosis resistance are not known, but hydrophobic bile acids, other agents that generate ROS/RNS, and actual components of a high-fat diet may have been key players.

Bile acids, inflammation and colon cancer

Numerous epidemiological studies have shown that fecal bile acid concentrations are increased in populations with a high incidence of colon cancer, suggesting an etiological linkage.[242–251] We have shown that supplemental feeding of deoxycholate in a mouse model can cause colitis in association with markers of oxidative stress.[115] An important link between high bile acid concentrations and colon cancer may, therefore, be the induction of inflammation, a condition that is associated with many cancers of the GI tract. Bile acids may induce inflammation through a disruption of tight junctions between colonic epithelial cells[115] and increase in transepithelial permeability,[88] providing a means for entry of pro-inflammatory bacterial species.[252] In addition, bile acids may cause dysregulation of cytokine expression through the constitutive activation of NF-κB,[98] a redox-associated transcription factor, that contributes to apoptosis resistance and a persistent increase in pro-inflammatory proteins.

Field defects in the colon

A "field defect" or "field of cancerization" is a region of tissue that precedes and predisposes to the development of cancer.[253] Field defects are of interest because they provide insight into the early events of progression to cancer and

may lead to clinically useful biomarkers of cancer risk. It is generally considered that a solid tumor is initiated by a series of somatically inherited changes (ie, aneuploidy, mutations or transmissible epigenetic events (epimutations), such as methylation of CpG islands[254]). Aneuploidy, mutations and epimutations are considered to contribute to early progression, usually by causing a somatically inherited proliferative advantage relative to surrounding cells. Furthermore, a mutator phenotype or genetic instability may accelerate the process of mutation followed by Darwinian selection.[19–21] In a normal population of dividing cells within a tissue, a cell may acquire a proliferative advantage through aneuploidy, mutation or epimutation. Such a cell will tend to undergo clonal expansion and replace neighboring cells, thus giving rise to a patch of abnormal cells. Within this patch, a cell might acquire a second aneuploidy, mutation or epimutation that provides a proliferative advantage compared to other cells within the established patch. This cell may then expand clonally forming a secondary patch within the first patch. Within this new patch, the process of mutation and Darwinian selection may be repeated several more times over a long period, perhaps decades, until a malignant cell arises that clonally expands into a cancer. If solid tumors generally arise by such a process, then tumors should ordinarily be accompanied by a field defect in associated tissue that may appear histologically normal.

The most common type of colorectal cancer, sporadic adenocarcinoma, develops by a pathway that appears to proceed through the following stages: normal flat mucosa, development of fields of defective flat mucosa, aberrant crypt foci, microadenomatous fields, adenoma with low grade dysplasia, adenoma with high grade dysplasia, and adenocarcinoma. However, currently, it is unclear whether all sporadic adenocarcinomas progress through each of these stages.

The morphologically normal appearing colonic mucosa of patients with colorectal neoplasia has been found by proteomic analysis to have numerous changes in protein expression.[255] Alterations of gene expression that occur in the normal appearing colonic mucosa of human colon cancer patients correspond to alterations observed in the normal appearing colonic mucosa of cancer prone APCmin mice.[256] The promoter of the DNA repair gene O^6-methylguanine-DNA methyltransferase (MGMT) is methylated in 46% of colonic tumors. Within this group of tumors with MGMT methylation, 94% of tissue samples from apparently normal mucosa associated with these tumors also had MGMT promoter methylation.[257] Often MGMT methylation was detected as far away as 10 cm from the tumor, indicating a field defect. Also patients with resected adenomas of the colon or rectum had altered expression of O-acetylated sialic acids in their "uninvolved" colon and rectal mucosa compared to sialic acids of colon or rectal mucosa of patients without colon tumors.[258] These findings indicate a field defect of sialic acid expression in the patients with colorectal cancer. Since a decrease in the level of O-acetylated sialic acids on the surface of colon epithelial cells is found to be associated with the early stages of colorectal cancer,[259] it is considered a risk factor. This decrease in the level of O-acetylated sialic acids may lead to an increase in the expression of sialyl LewisX, a tumor-associated antigen related to the progression of colorectal cancer cells to metastasis.[260]

An imbalance of proliferation and apoptosis in the left colon and sigmoid/rectum was found in patients who had a resected large adenoma ($\geq$1.5 cm) compared to patients who never had a tumor.[261] Several lines of evidence indicate that colorectal cancers often arise in a field of apoptosis-resistant cells. The Bcl-x_L protein inhibits apoptosis, in part, by inhibiting release of cytochrome c from the mitochondria. The expression of Bcl-x_L was found to be increased at 1 cm and 10 cm away from colorectal adenocarcinomas in the non-neoplastic colorectal mucosa, suggesting the presence of an apoptosis-resistant field.[262] The prototypic anti-apoptotic protein, Bcl-2, also behaves similarly.[263] Expression of the antiapoptotic *survivin* mRNA in colon tumors predicts poor patient survival due to subsequently recurrent colorectal tumors.[264] The normal appearing mucosa of about half of patients with survivin-positive tumors also express this mRNA. This finding suggests that *survivin*- positive tumors often develop in a normal appearing, but *survivin*- positive, apoptosis-resistant field. When rats are fed a diet containing 0.2% cholic acid, their colon crypt cells develop increased resistance to apoptosis.[240] Apoptosis-resistant crypts may then spread through the flat colonic mucosa by crypt fission.[265] These various observations suggest that repeated exposure of the colon to high levels of bile acids as a result of a Western style diet cause Darwinian selection for cells resistant to induction of apoptosis, and give rise to fields of apoptosis-resistant epithelium that may further evolve to malignancy.

Work from our laboratory on biomarkers associated with colon cancer risk indicate that, in addition to loss of apoptosis competence, lectin staining using DBA (*Dolichos biflorus agglutinin*) (a measure of epithelial cell differentiation), cytochrome c oxidase subunit I (CcOI) (a mitochondrial DNA-encoded subunit of CcO) and Pms2 (a DNA repair protein) were all significantly decreased in the normal-appearing flat mucosa of patients with colon cancer compared to that of control subjects.[213,241,266]

Scalmati and Lipkin [267] reviewed numerous studies on cell proliferation pattern in patients with colon adenomas or cancer. Expansion of the proliferative compartment (a lumenward displacement of the zone of active cell proliferation in the crypts) occurred along the entire colon of individuals, indicating that there was increased risk throughout the colon, no matter where the actual lesion was located. We also determined that apoptosis resistance in the nonneoplastic flat mucosa of patients with colon cancer was present in several regions of the colon far removed from the tumor site,[45] indicating a generalized field defect of apoptosis resistance, albeit "patchy" in nature,[213] throughout the colon.

A recent study indicated that the consumption of a Western style diet plays a crucial role in the progression of a field defect to colon cancer.[268] Among 1009 patients with resected stage III colon cancer, colon cancer recurred in 324 patients in a median of 5.3 years. This recurrence suggests the frequent presence of a field defect. The patients had all undergone fluorouracil-based adjuvant chemotherapy after surgery, and were evaluated for diet during and 6 months after adjuvant chemotherapy. The rate of cancer recurrence was almost threefold greater among patients in the highest quintile of Western style diet consumption compared to those in the lowest quintile of Western style diet consumption.

Prevention of bile acid-induced stresses and genomic instability: importance of butyrate

We have reviewed evidence that deoxycholate induces cellular stresses in epithelial cells of the GI tract and that bile acids play a role in colon carcinogenesis. Although hydrophobic bile acids were once believed to be mainly tumor promoters, the bile acid induction of DNA damage and aneuploidy indicates that they are also genotoxins relevant to the initiation and progression of the neoplastic process. It is well documented that phytosterols and other types of antioxidants, micronutrients and dietary fiber have antineoplastic effects. An overall discussion of dietary-related colon cancer chemopreventive agents is beyond the scope of this review. However, we will focus on the short-chain fatty acid, butyrate, since previous studies have shown that butyrate interferes with many deleterious effects of hydrophobic bile acids such as increased proliferation,[269–272] and has been shown to have anti-genotoxic effects on deoxycholate [143] and H_2O_2-induced[273,274] genotoxicity in colon epithelial cells. Furthermore, butyrate affects many signal-transduction pathways and histological parameters associated with colon carcinogenesis.[272,275–286]

Butyrate is a 4-carbon fatty acid that is an end product of bacterial fermentation of dietary fiber and occurs at concentrations of 2–10 mM in the colon.[287] Certain intestinal bacterial species (eg, *Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia, Coprococcus*) are butyrate producers[288–290] and beneficial to colon cancer prevention, whereas others, such as *Enterococcus faecalis* (extracellular superoxide and H_2O_2-producing bacterial species[291,292]) and *Desulfovibrio* (sulfate-reducing bacteria which produces hydrogen sulfide) are deleterious in that they cause DNA damage and chromosomal instability.[291–295] In a recent study that quantified specific bacterial species using real-time PCR in the feces of healthy control volunteers and patients with colon cancer, it was found that the butyrate-producing bacteria species were significantly decreased and the superoxide-producing bacterial species were significantly increased in the feces of colon cancer patients compared to control subjects.[290] It is significant that the feces of patients with ulcerative colitis, a pre-neoplastic inflammatory condition of the colon, uniformly contain sulfate-reducing bacteria. Sulfide substantially increases the proliferative index of the crypts in mucosal biopsies, and this increase is prevented by the presence of butyrate.[296] A general consequence of the fermentation of fiber is to reduce the pH of the gut lumen, thereby affecting bacterial metabolism and competition[297] and reducing the bacteria-mediated formation of hydrophobic bile acids.[298] These shifts in bacterial species may be important determinants of the risk of colon cancer.

It is significant that butyrate can protect against oxidative DNA damage induced by deoxycholate *ex vivo* in colonocytes derived from human biopsies.[143] The processes in the colon that are affected by butyrate include a suppression of proliferation,[271,277,299] induction of apoptosis,[214,215] induction of differentiation,[275,276,278] a reduction in inflammation,[280,286] suppression of NF-κB activation,[300] increase in xenobiotic metabolizing enzymes[301,302] and a decrease in DNA damage and genomic instability.[143,273,274] All of these processes affect colon carcinogenesis in a profound way.[280,303]

In our studies of field defects in the human colon, we observed that in the nonneoplastic flat mucosa of patients with colon cancer there is an increase in apoptosis resistance,[44,45,212,213] loss of differentiation,[213] decrease in mitochondrial gene expression (eg, CcOI)[266] and a decrease in PMS2, a mismatch repair protein.[241] It is significant that butyrate has opposite effects. It can increase differentiation, induce apoptosis, increase CcOI levels,[276] and has more potent antineoplastic effects on colon cancer cells defective in mismatch repair.[304]

Future directions

We have just begun to understand the role of hydrophobic bile acids in inducing DNA damage and genomic instability in colon epithelial cells, and potential strategies to reduce these deleterious effects. Some key areas that require further experimentation include: 1) the role of oxidative stress as mediators of the effect of bile acids on the reduction of DNA repair proteins (eg, Ku80, MSH3), tumor suppressors (eg, p53) and spindle checkpoint proteins (eg, Mad2); 2) the role of oxidative stress in the deoxycholate-induced induction of micronuclei and aberrant mitoses; 3) the ability of deoxycholate to induce aberrant mitoses and aneuploidy *in vivo* using mouse models; 4) the possible synergistic effect of bile acids and environmental agents (eg, nicotine from tobacco smoking, arsenic, food carcinogens) in inducing DNA damage and genomic instability; and 5) the effectiveness of dietary-related nutrients and co-factors (eg, zinc, niacin, selenium compounds, plant polyphenols, butyrate) in preventing the genotoxic effects of hydrophobic bile acids both *in vitro* using cell culture models and *in vivo* using mouse models. The results from these types of experiments will have relevance to other cancers of the GI tract (esophagus, stomach, liver, pancreas) in which hydrophobic bile acids have a potential etiologic role.

Summary

In this review, we have described the likely major mechanisms by which hydrophobic bile acids can induce stresses on cells, which can then result in colon carcinogenesis. Persistent exposure of colon epithelial cells to hydrophobic bile acids can result in the development of apoptosis resistance and the modulation of numerous genes/proteins associated with chromosome maintenance and mitosis. Hydrophobic bile acids also damage the genome through multiple mechanisms, including oxidative DNA damage, p53 and other mutations, micronuclei formation and aneuploidy, all major components of genomic instability. These findings link dietary-related factors to the development of genomic instability, extend our understanding of the underlying causes of colon cancer, and may open up new avenues for hypothesis-driven biomarker development to assess colon cancer risk.

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