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World Journal of Gastrointestinal Oncology (*World J Gastrointest Oncol*, *WJGO*, online ISSN 1948-5204, DOI: 10.4251) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJGO covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Genotypic characteristics of resistant tumors to pre-operative ionizing radiation in rectal cancer

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Abstract

Due to a wide range of clinical response in patients undergoing neo-adjuvant chemoradiation for rectal cancer it is essential to understand molecular factors that lead to the broad response observed in patients receiving the same form of treatment. Despite extensive research in this field, the exact mechanisms still remain elusive. Data raging from DNA-repair to specific molecules leading to cell survival as well as resistance to apoptosis have been investigated. Individually, or in combination, there is no single pathway that has become clinically applicable to date. In the following review, we describe the current status of various pathways that might lead to resistance to the therapeutic applications of ionizing radiation in rectal cancer.

Key words: Ionizing radiation; DNA double-strand break; Non-homologous end-joining pathway; DNA-PKcs; Ku proteins; Complete pathological response; Radiation therapy; Apoptosis; Angiogenesis

Core tip: Treatment of locally advanced rectal cancer stage Ⅱ and Ⅲ includes neoadjuvant chemo-radiation followed by surgery if clinically feasible. A strategy of observing patients without an operation has been proposed by some surgeons, but this is still the center of much debate. Moreover, the therapeutic effect of ionizing radiation in treatment of rectal cancer varies significantly from one person to another. This has led investigators to identify the molecular targets and pathways in rectal tumors resistant to ionizing radiation in a bid to improve the therapeutic effect of radiation by advanced biomedical and genetic engineering.

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INTRODUCTION

There are approximately 40340 patients diagnosed with rectal cancer annually in United States[1]. Cancer of the colon and rectum combined claimed 51690 deaths in 2012[1]. Rectal cancer, though staged similarly to colon cancer, is managed differently due to the pelvic location of the rectum. The rectum is in close proximity to the urogenital organs and anal sphincters. Hence, surgery for rectal cancer is associated with complications ranging from 15% to 70%[2]. Moreover, many patients will have local as well as distant metastasis during post-op surveillance[3,4]. Hence, careful and methodical planning

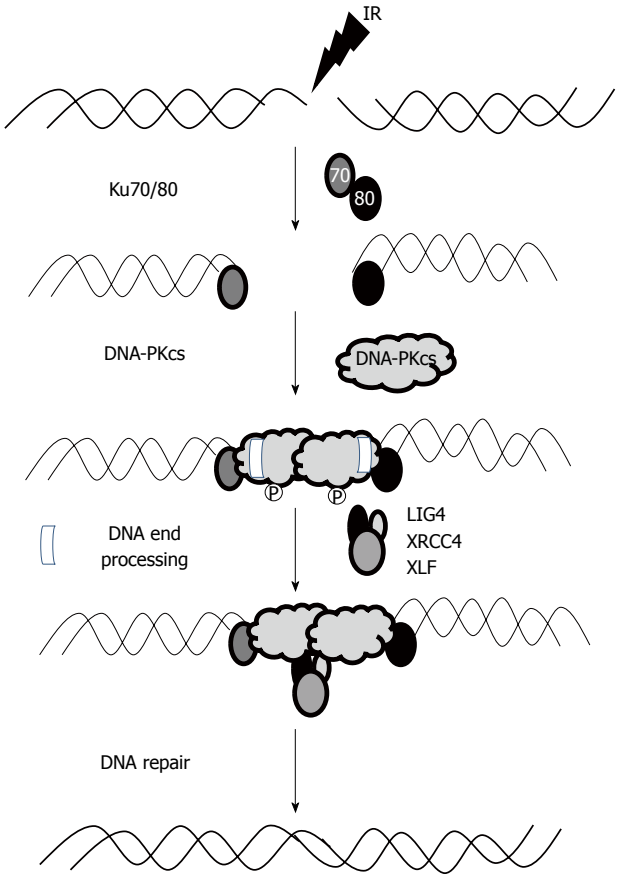

Figure 1 Schematic representations of double-strand break repair by non-homologous end-joining mechanism. The KU proteins are the initial participants in this process as they rapidly bind to broken DNA segments. Another major function of the KU proteins is the active recruitment of DNA-PKcs. DNA-PK activation assists with the recruitment of other proteins involved in the limited DNAend-processing (Artemis, pol m, pol l, and TDK) required to generate ligatable DNA ends. Ligation is mediated by the LIG4/XRCC4 complex and is assisted by the ligation mediator XLF. Once this process is completed, DNA integrity is maintained.

is required to avoid unnecessary surgery with potential short and long term complications. Recent studies have underscored the importance of ionizing radiation (as neoadjuvant therapy) in patients with stage Ⅱ and Ⅲ rectal cancer. There are many benefits to the use of IR in the neoadjuvant compared to the adjuvant setting[5]. Additionally, in some cases, this approach allows the tumors to be down-staged resulting in complete pathological response (pCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation at laparotomy) or complete clinical response (cCR, *i.e.*, complete obliteration of the tumor following preoperative chemoradiation during repeat colonoscopy or other diagnostic modalities such as MRI).

However, the benefit from preoperative radiation varies significantly in trials with a substantially wide pCR (9%-37%)[6-10]. Patients who achieve a pCR have better outcomes compared to patients who do not[11]. Some surgeons have elected a watchful waiting approach for patients who achieve cCR[12-17].

The logical clinical and pre-clinical question is to devise methods by which we can personalize treatment for rectal cancer, such that the most effective therapy with the least side effect profile can be offered consistently to patients affected by rectal cancer. In order to achieve this objective, extensive research has been performed over the last few decades to identify biological markers and genetic phenotypes that can predict successful response to radiation and translate into improved survival. We present a review of the current status of these markers.

THE THERAPEUTIC EFFECTS OF IR

The NHEJ pathway of DNA repair

The therapeutic effect of IR is largely the result of double stranded DNA breaks that result from IR-induced DNA damage. DNA breaks are difficult to repair and typically result in apoptosis. DNA double-strand break (DSB) can be repaired by one of the following three pathways: homologous recombination[18], non-homologous end-joining (NHEJ) pathway, or an alternate NHEJ pathway (characterized by larger deletions and translocations)[19]. The details behind the selection and execution of these pathways are not entirely clear, but it seems that NHEJ is the major pathway as it is the only one that occurs in all stages of cell cycle.

The NHEJ pathway is essential for DSB repair and is also important for V (D) J recombination during T and B cell lymphocyte development. The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is an integral part of the NHEJ pathway. The actual mechanism of this pathway is rather complex (Figure 1), but can be broadly classified into three steps. In the first phase, Ku 70/80 heterodimer identifies DSB, facilitates the activation and recruitment of DNA-PKcs, and then ties the DNA ends in a synaptic complex[20]. The next step involves enzymatic processing of the DNA ends followed by ligation (by DNA ligase Ⅳ) in the last phase. The order and timing of this sequence of events is not well defined; however, it is widely regarded that Ku 70/80 protein is the most important and integral part of this sequence as it recruits DNA-PKcs as well as interacts with a host of other important proteins. Moreover, Ku has lyase activity allowing it to process DNA ends during NHEJ[21].

Following successful DNA repair, the cell might undergo back to the normal cell cycle. If some error occurs during the repair, the cell might undergo genomic instability and if the cell is unable to repair the radiation-induced damage, it undergoes apoptosis (Figure 2)[22]. Thus, a logical place to begin investigating marker of radioresistance is by interrogating the NHEJ pathway of DNA repair in cancer cells.

Role of DNA-PKcs

DNA-PKcs has multiple roles in DNA repair and carcinogenesis. DNA-PKcs facilitates DSB repair, thus ensuring stability and integrity of genetic chromosomes. Hence, low levels of DNA-PKcs might result in muta-

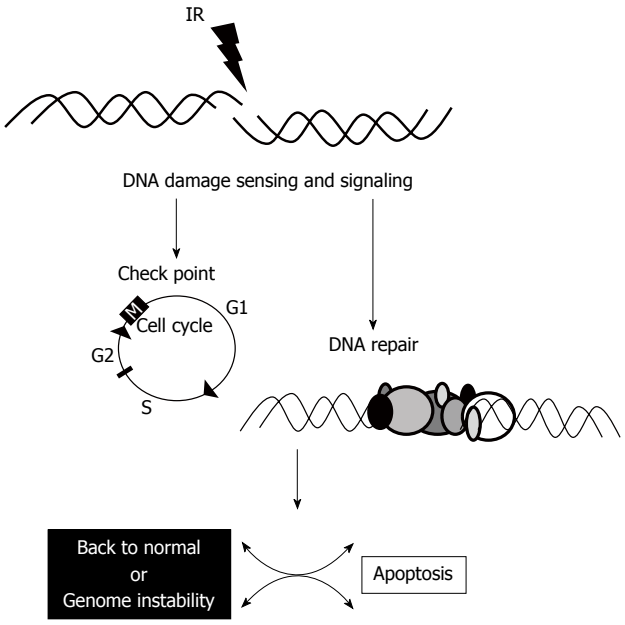

Figure 2 Schematic representation of the events that occur following IR-induced DNA damage. Sensing mechanisms and signaling first stop the cell cycle that allows the cell to repair the DNA damage. If unsuccessful, apoptosis ensues. If the repair is nearly complete, the cell might continue to replicate with genome instability.

tions promulgating the cascade of carcinogenesis. A cell with low levels of DNA-PKcs might be unable to repair the DNA damage incurred by IR and destine the cell for apoptosis. In this scenario, low levels of DNA-PKcs should be a surrogate for radiosensitivity.

On the other hand, cancer cells might contain higher DNA-PKcs levels induced by the rapid cell turnover. In this scenario, increases in DNA-PKcs activity will enhance cancer cell resistance and decrease susceptibility to chemotherapy and ionizing radiation[23-26].

Pre-clinical studies have demonstrated that DNA-PKcs deficient Chinese hamster ovary cells showed profound cell death following treatment with IR compared to the DNA-PKcs complimented V3-YAC cells[27]. Colon cancer HCT-116 DNA-PKcs$^{-/-}$ cells and xenografts were exquisitely sensitive to IR[28,29]. Unfortunately, the role of DNA-PKcs activity in development of various cancers has been investigated in multiple studies and has shown conflicting results in carcinogenesis as well as being a poor predictor of a response to IR, but more data is needed in this area (Table 1).

Significant increases of DNA-PKcs activity have been observed in certain gastrointestinal cancers such as colorectal cancer[30,31], esophageal cancer[32], nasopharyngeal cancer, and non-small cell lung cancer[33]. Conversely, loss of DNA-PKcs expression has been linked to gastric tumors correlating with signs of invasion and poor survival[34,35].

Levels of DNA-PKcs in cancer cells before treatment (radiation or chemotherapy) has been compared to levels after treatment, and have shown mixed results. The expression of DNA-PKcs was noted to be directly proportional to a favorable response with radiation in esophageal and early breast cancer but not in nasopharyngeal cancer[36-38]. On the other hand, studies have revealed increased levels of DNA-PKcs and Ku proteins in residual tumors after radiation treatment, suggesting a means of survival and a marker of radioresistance in recurrent tumors[39].

While the cellular status of the DNA-PKcs as a predictor of IR remains to be investigated, DNA-PKcs inhibition might have a therapeutic role in rectal cancer. Pre-clinical studies showed that pharmacological inhibition of DNA-PKcs led to substantial chemo- and radiosensitization[27,40-42]. The effect of DNA- PKcs inhibitors has been examined in mouse xenograft tumor models with favorable results. There has been significant tumor growth delay and improved survival in mice treated with combined DNA-PKcs inhibition and ionizing radiation. The combination treatment reduces levels of cell proliferation marker Ki67 and increases activity of certain proteins known for its anti-tumor properties[43,44].

Inhibitors of DNA-PKcs have been shown to have a synergistic effect along with cisplatinum/platinum based drugs in treatment of ovarian, colon, and breast cancer[45-47]. Multiple DNA-PKcs kinase activity inhibitors are not only in various stages of development but a few are being tested in clinical trials (Table 2). Similarly, new *in vivo* substrates of DNA-dependent protein kinase (Akt1/PKBa, Hsp90a, NR4A[48-52]), which can be induced by ionizing radiation have been identified.

Furthermore, additional DNA-PKcs inhibitors have been developed such as anti-DNA-PKcs ScFv 18-2 (derived from an existing anti-DNA PKcs monoclonal antibody)[53], and anti-DPK3-scFv (selected from a humanized semi-synthetic scFV library)[44]. These anti-DNA PKcs sensitize cells to radiation induced injury[44,54,55] in a similar fashion to RNA inhibition of DNA-PKcs transcripts[56-58].

The interaction between epidermal growth factor receptor (EGFR) and the DNA-PKcs has also been explored. This interaction is required for radiation induced nuclear AKT phosphorylation and cell survival[52,59,60]. Similarly, blockage of EGFR signaling pathway with a monoclonal antibody can inhibit DNA-PKcs activation and thereby decrease DNA repair capacity. This could enhance sensitization and susceptibility of cells to ionizing radiation[61,62].

Clinically, deficiency in DNA-PK activity led to sensitivity to nitrogen mustards in patients with chronic lymphocytic leukemia[25]. The drug 2-N-morpholino-8-dibenzothiophenyl-chromen-4-one (NU7441) is a potent and specific DNA-PK inhibitor[63]. Treatment with NU7441 and topoisomerase inhibitors combined with IR caused potent chemo-radio sensitization in SW620 colorectal cancer cells as well as xenografts[27]. The various mechanisms by which DNA-PKcs inhibitors facilitate radiation induced death include apoptosis[64,65], acceleration of senescence, induction of mitotic catastrophe, and autophagy[43,66,67].

Studies evaluating expression of DNA-PKcs in pe-

Table 1 Association between DNA-PKcs activity and cancer development from clinical investigations

Tumor type	Assay	Specimen	Sample size	DNA-PKcs activity	Interpretation
Nasopharyngeal cancer	IHC	Tumor	66	↑ in 70% of tumor tissue	No association with locoregional control and survival
Nasopharyngeal cancer	IHC	Tumor	223	↑ in 37% of tumor tissue	Overexpression associated with advanced stage and poor survival
Esophageal cancer	IHC, IB, Kinase activity	Tumor, normal	13 paired	↑ in tumor tissue	NA
Gastric cancer	IHC	Tumor	279	↑ in 73% of tumor tissue	Loss of expression associated with lymphatic invasion, lymph node metastasis, advanced pathological stage, and poor survival
Gastric cancer	IHC	Tumor, normal	791	↑ in 80% of tumor tissue	Loss of expression associated with intratumoral neutrophils, microsatellite instability, mutations in DNA-PKcs and poor survival
Colorectal cancer	RT-PCR, IB, kinase activity	Tumor, normal	12 paired	↑ in tumor tissue	NA
Colorectal cancer	IHC, IB	Tumor, normal	359 (35 paired)	↑ in 64% of tumor tissue	Overexpression associated with clinical stage, lymphatic invasion, distant metastasis and poor survival
Non-small cell lung cancer	IHC	Tumor	113	↑ in 89% of tumor tissue	Overexpression associated with tumor grade
Non-small cell lung cancer	IHC	Tumor	86	↑ in 87% of tumor tissue	No association with clinical characteristics or outcome
Non-small cell lung cancer	RT-PCR	Tumor, normal	140 paired	↑ in tumor tissue	Overexpression associated with poor survival
Non-small cell lung cancer	IHC	Tumor, normal	116 (12 paired)	↑ in 75% of tumor tissue	No association with clinical characteristics or outcome
Glioma	Kinase activity	Tumor	36	↑ in tumor tissue	Hyperactivity correlates with rumor grading
Ovarian cancer	IHC	Tumor, normal	100	↓ in 40% of tumor tissue	loss of expression associated with tumor progression, advanced clincal stage, and lymph node metastasis
ALL, CLL, lymphoma, multiple myeloma	IHC, IB	Lymphoid tissue	86	↑ During lymphoid development and in lymphoid malignancies	Overexpression associated with higher lymphoma grading and degree of maturation in lymphoid malignancies other than multiple myeloma
B-cell CLL	IB, kinase activity	Lukemia cells	54	↑ in del(17p) and del(11q)	Overexpression associated with shorter treatment free interval
B-cell CLL	RT-PCR	Lukemia cells	50	↑ in del(17p)	Overexpression associated with poor survival
Cancer of breast, cervix, head and neck esophageal and lymphoma	Kinase activity	PBLs	167	↓ in advanced stage	Hypoactivity associated with advanced stage and distant metastasis
Radiation response					
Esophageal cancer	IHC	Tumor	67	↑ in 54% of tumor tissue	Overexpression predicts better response to chemoradiation
Oral squamous cell carcinoma	IHC	Tumor	42	↑ in residual tumor after RT	Not predictive of radiation response
Cervical cancer	IHC	Tumor	22	↑ in residual tumor after RT	No association with clinical characteristics
Breast cancer	IHC	Tumor	224	↑ in 43% of tumor tissue	Overexpression predicts better locoregional control of radiation alone versus chemotherapy alone in early stage
Cancer risk					
Lung cancer	Kinase activity	PBLs	Cancer 41/healthy 41	↓ in cancer patients	Hypoactivity associated with cancer of the lung
Breast, cervix, head and neck, esophagus and lymphoma	Kinase activity	PBLs	Cancer 93/healthy 41	↓ in cancer patients	Hypoactivity associated with chromosomal instability and cancer of breast and cervix

Adapted with permission[148]. ALL: Acute lymphocytic leukemia; CLL: Chronic lymphocytic leukemia; IHC: Immunohistochemistry; PBLs: Peripheral blood lymphocytes; RT-PCR: Reverse transcription polymerase chain reaction; ↑: Indicates increase activity; ↓: Indicates decrease activity.

ripheral blood lymphocytes (PBLs) as a marker of host immunity and cancer development have shown an additional role in cancer development as it relates to host immunity. Data from multiple studies demonstrated that cancer patients have a lower level of DNA-PKcs activity in PBLs[23,68,69], suggesting impaired ability to recognize cancer cells leading to a poor prognosis. Whether this is mediated by activation of natural killer (NK) cells or release of pro-inflammatory cytokines is not clearly understood[70]. Destruction of NK cells leading to increases

Table 2 Non-homologous end-joining inhibitors

Inhibitor	Mechanism/comments
A12B4C3	PNKP inhibitor, sensitizes cells to camptothecin
BTW3	A small peptide DNA-PK inhibitor, proposed to compete for DNA-PKcs autophosphorylation
KU0060648	DNA-PK and P13K inhibitor
NU7441/KU57788	DNA-PK inhibitor, competitive with ATP
ScFv 18-2	An antibody-derived DNA-PK inhibitor that can bind to an epitope unique to DNA-PKcs
ZSTK474	DNA-PK and P13K inhibitor, competitive with ATP; in phase 1 clinical trials (NCT01280487 and NCT01682473)
CC-115	Dual inhibitor of DNA-PKcs and mTOR, in phase 1 clinical trials
CC-122	DNA-PK inhibitor, in phase 1 clinical trials

Reprinted with permission from Elsevier[149]. P13K: Phosphatidyl inositol 3 kinase.

in spontaneous tumor development in mouse models[71] leans in favor to the former hypothesis. Moreover, an inverse association between DNA-PKcs activity in PBLs and stage of cancer was also observed in patients who were treated with radiotherapy for advanced cancer, displaying poorer prognosis and higher frequency of distant metastasis[68].

In addition to its role in NHEJ pathway, DNA-PKcs regulates the DNA damage repair mechanisms by a variety of mechanisms. These include DNA interstrand crosslink (ICL) repair[72,73], AKT activation, EGFR nuclear translocation, or activation/mobilization of chromatin remodeling factor structure-specific recognition protein 1 (SSRP1) from nucleolus[60,74,75]. Biomedical engineering aiming to mimic some of the activities of the DNA-PKcs has been instrumental in developing novel agents that might be useful for cancer therapeutics.

It is clear that the status of the DNA-PKcs plays a fundamental role in ionizing radiation-induced cell death. Many aspects of its role in cancer therapeutics are currently under investigation. In rectal cancer, the role of DNA-PKcs is still in its infancy. As markers of a response to ionizing radiation, the role of the DNA-PKcs is complicated by the fact that there is paucity of high quality data. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below). In prostate cancer, nuclear positivity for DNA-PKcs was associated with chemical recurrence[76]. Further studies are required to shed more light into these issues.

The Ku proteins

Ku70 and Ku80 proteins are essential components of the NHEJ pathway. These proteins serve as a medium by which multiple other DNA-repair proteins can be attached to the pathway cascade[77]. Importantly, the Ku proteins have a high affinity for broken DNA strands and rapidly bind to them. This initial process also recruits DNA-PKcs for DNA repair, though the exact mechanism is still unknown[78,79]. Additionally, Ku proteins play a major role in recruitment of XRCC4[80,81], XLF[82], APLF (APTX and PNK-like factor)[83] to DSBs helping with the repair process and promoting NHEJ. Moreover, Ku has the ability to enzymatically process DNA ends during NHEJ using the 5'-deoxyribose-5-phosphate (5'-dRP)/AP lyase activity[21]. Ku also excises abasic sites near DSBs suggesting a potential role in repairing damage by IR[21].

Intuitively, tumors that express high levels of Ku proteins should be able to repair the damage induced by IR more efficiently and thus become more resistant to therapy. *In vitro* studies have failed to show an association between the Ku proteins and radiosensitivity[84]. *Ex vivo* studies have also interrogated the role of the Ku proteins as surrogates of a response to IR.

Lack of Ku70 immunoreactivity correlated with radiosensitivity in patients with carcinoma of the cervix. In these patients, survival was better in tumors that had lower nuclear expression of Ku70[84]. In squamous cell carcinoma of the head and neck, Ku80 over expression was an independent predictor of regional recurrence and mortality in patient treated with IR[85]. Similarly, in rectal cancer low levels of Ku70 and Ku80 were associated with pCR. Ku70 was associated with down-staging. Disease free survival was 42% in patients with high Ku70 expression compared to 78% in patients with low expression of the same protein. Similar results were observed for Ku80[86]. Elevated levels of Ku proteins occur in high grade lymphoid malignancies[87]. The Ku70/Ku80 heterodimer DNA end-binding activity was 2- to 3-fold higher in the resistant B-CLL cell subset compared with the sensitive B-CLL cell subset[88], highlighting a possible mechanism behind increased DNA-PKcs activity in resistant CLL cells. The authors showed that novel DNA-dependent protein kinase (DNA-PK) inhibitor, NU7026 (2-(morpholin-4-yl)-benzo[h]chomen-4-one), and the phosphatidylinositol 3 (PI-3) kinase inhibitor, wortmannin, restored sensitivity to DNA damage-induced apoptosis of otherwise resistant cells.

Ku proteins can be upregulated after radiation treatment[39,89]. In one such study, expression of DNA-PK complex proteins (including Ku 70 proteins) increased after radiation treatment in residual tumors, and the increased values correlated with the tumor radiation resistance[89]. Various mechanisms have been postulated behind the role of Ku proteins in radioresistance. A distinct cell-interdependent signal is conveyed through gap junctions during chemotherapy with cisplatin, mediated by

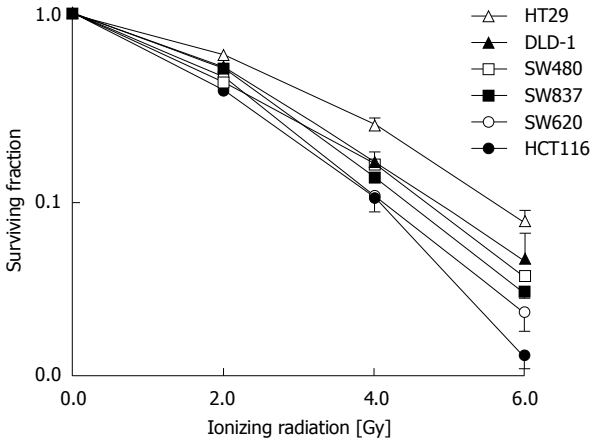

Figure 3 Response to ionizing radiation in several colorectal cancer cell lines subjected to various doses of ionizing radiation. There is a variable response to the same doses or ionizing radiation (Gy).

the kinase function of Ku70, Ku80 and DNA-dependent protein kinase complex. This communication may explain the resistance to cisplatin-induced death of cancer cells[90]. It is also possible that the role of Ku proteins and DNA-PKcs in DNA damage repair depends upon the extent and complexity of damage by IR. Studies have revealed that simple DSBs induced by laser irradiation are repaired rapidly involving Ku70/80 and XRCC4/Ligase IV/XLF. In contrast, DSBs with greater chemical complexity are repaired slowly and requires additional use of DNA-PKcs[91].

While these data seem compelling, more research is required prior to establishing the role of the Ku proteins in a response to radiation in rectal cancer. Current data on this subject, while promising, is currently limited and not clinically available. In rectal cancer, our group demonstrated counter-intuitive results with regards to the role of DNA-PKcs in the response to IR (discussed below).

ANALYSIS OF GENOTYPIC ORIGINS OF RADIORESISTANCE *IN VITRO* AND *IN VIVO* MODELS OF RECTAL CANCER

Examination of factors leading to radioresistance can practically be approached *in vitro*. Analysis of five colon cancer cell lines (HT29, DLD-1, SW480, SW620, and HCT116) as well as one rectal cancer cell line (SW837) have demonstrated a similar pattern of response to a group of patients treated for rectal cancer with pre-operative IR (Figure 3)[92]. The cell lines that have been treated with IR and examined originate from patients with different characteristics.

SW480 cells were derived from a primary Duke's stage B colon adenocarcinoma from a 50-year-old Caucasian male, while the SW620 cell line was cultured from a lymph node metastasis from the same patient at a later time. The DLD-1 cell line was established from an adult male with adenocarcinoma of the colon. The SW837 cell line was derived from a 53-year-old Caucasian male with rectal cancer. HCT-116 cells were cultured from an adult male with colon cancer. HT-29 cells were derived from a 44-year-old Caucasian woman with colorectal adenocarcinoma. All of these cells have mutations of the p53 gene, except for HCT-116 cells (p53-Wt). HT-29 cell have mutations of both alleles of the p53 gene (p53-null)[92]. HCT-116 cells display microsatellite instability.

These cells have been extensively studied and a number of properties are known. Analysis of these factors and a response to IR has not yielded any uniform patter of predictability that could be surrogate markers in *ex vivo* studies. For instance, the inhibitor of apoptosis, survivin, has been shown to play a significant role in resistance to IR (discussed below)[93]. Analysis of this model of rectal cancer *in vitro* (Figure 3) has not consistently corroborated this finding. For instance, survivin was expressed in higher levels in the radiosensitive SW620 compared to the relative more radioresistant SW480 cell line. Interestingly, these two cells originated from the same patient one at the time of stage Ⅱ colon cancer (SW480) and the second one from a lymph node metastasis (SW620) such that these two cell lines contain similar genetic background.

Analysis of these cell lines is representative of the response that was observed in 117 patients who were treated with preoperative ionizing radiation and underwent surgical resection (Figure 4). A pivotal question is to determine what causes these differences in patients and cell lines receiving the same treatment. A simple approach in the laboratory is to take the more radioresistant and the more radiosensitive cells and analyze specific differences. This approach has been undertaken *in vitro* and *in vivo*. HCT-116 cell and xenografts are substantially more sensitive to IR compared to HT-29 cells and xenografts (Figure 5).

DNA repair in this model

Analysis of DNA induced damage (by γH2AX) indicated that the radioresistant HCT-116 cells suffer more DNA damage when exposed to IR and that this damage persists over time indicating a poor ability of the cells to repair the DNA affected by IR (Figure 6)[94]. Predictably, HT-29 cells should be able to repair DNA more effectively and should have increased levels of DNA-PKcs and Ku proteins. In fact, the opposite results have been observed in our studies. Our results showed that compared to HCT-116 cells, HT-29 cells expressed lower levels of DNA-PKcs and Ku proteins[95].

Cell cycle kinetics in this model

Examination of cell cycle kinetics demonstrates that the radiosensitive HCT-116 cells substantially accumulate in the G-2 phase of the cell cycle. HT-29 cells proceed through the cell cycle in spite of receiving the same dose of IR (Figure 7)[22,28,92,94,96,97]. According to these observations, there should be differences in cell cycle regulators and apoptotic factors that could be used to predict a response to IR.

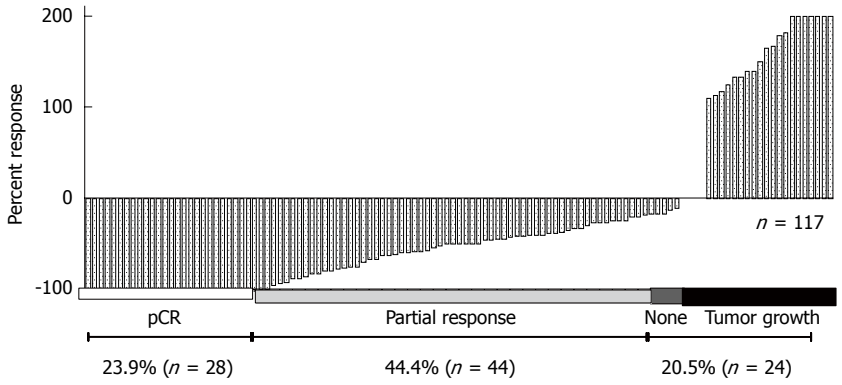

Figure 4 There is a high variability of a response to ionizing radiation in rectal cancer patients treated with pre-operative ionizing radiation. Each bar on the X-axis represents an individual patient. The Y-axis represents the clinical response to pre-operative ionizing radiation. Nearly one fourth of patients achieve a pCR, but close to another fourth do not respond to the same form of treatment, while the rest of patients have achieve a partial response.

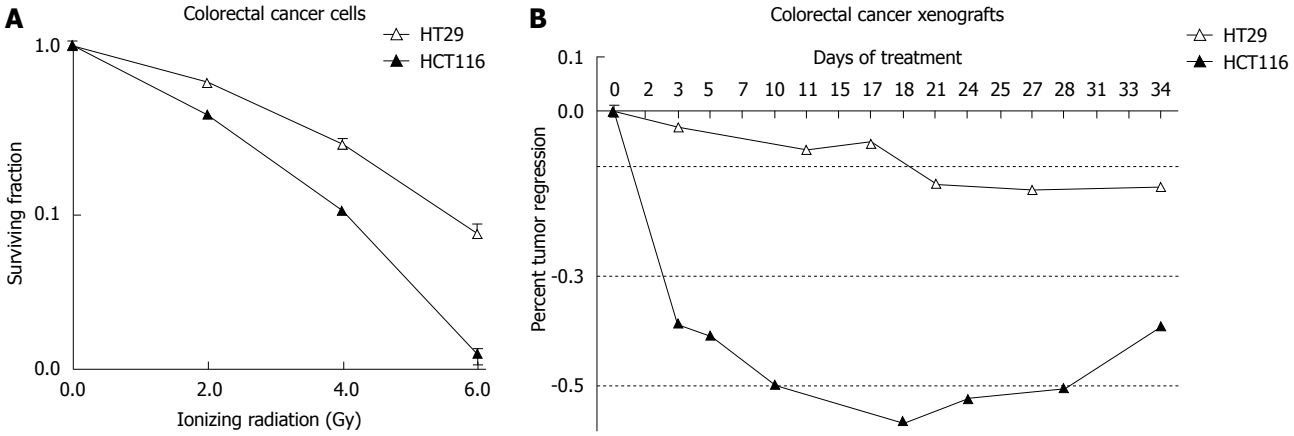

Figure 5 Analysis of the most radiosensitive (HCT116) and the most radioresistant (HT29) cells (A), a similar response has been noted in cells implanted in immune compromised mice bearing xenografts of these cells (B).

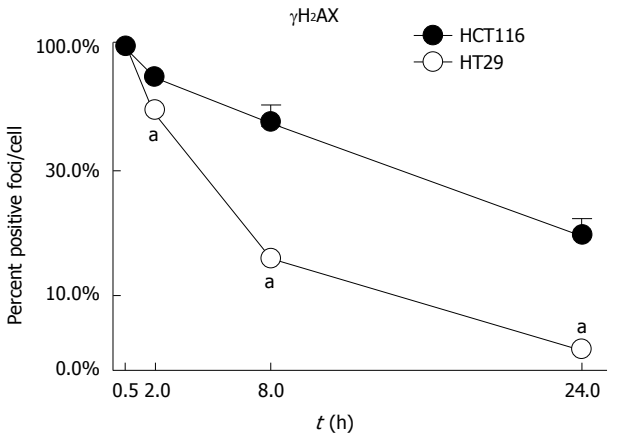

Figure 6 Analysis of DNA-induced damaged by ionizing radiation as determined by γH2AX. HCT116 cells undergo more pronounced damage when treated with the same dose of ionizing radiation compared to HT29 cells. The damage induced in HCT116 cells persists over time. [a]$P < 0.05$ *vs* HCT116.

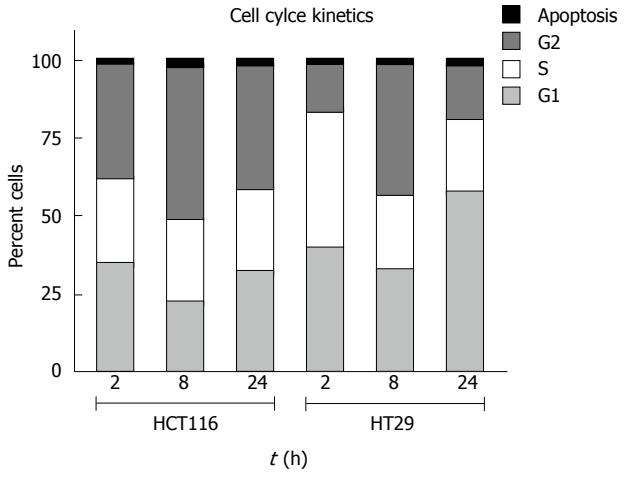

Figure 7 Cell cycle kinetics of HCT116 and HT29 cells treated with 2.0 Gy ionizing radiation. There is a pronounced accumulation of cells in G2 in HCT116 cells. HT29 cells continue through the cell cycle in spite of receiving the same dose of ionizing radiation.

Apoptosis in this model

Analysis of this model with regards to the central mediators of apoptosis (as depicted in Figure 8) has demonstrated the following in HCT116 (*vs* HT29 cells): marked over expression of p21, decreased expression of p53, Bax, Bcl-2 and survivin[92]. Examination of these findings is intuitive in some areas while counterintuitive in oth-

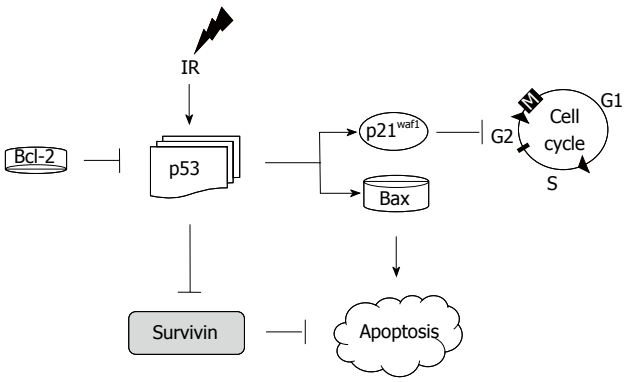

Figure 8 Schematic representation of molecular events following the cellular response to ionizing radiation-induced damage. Ionizing radiation causes an up-regulation of p53. p53 then directly activates the cyclin dependent kinase inhibitor p21. Cell cycle progression stops until the cell repairs the damaged induced by ionizing radiation. If the cell is unable to repair itself, it undergoes apoptosis. Bcl-2 inhibits p53 up-regulation, while p53 inhibits the inhibitor of apoptosis: survivin.

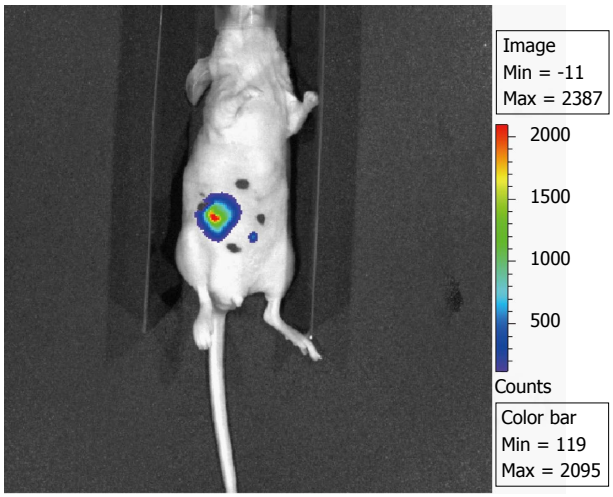

Figure 9 Orthotopic model for the study of rectal cancer. This model has the following characteristics: (1) cecal transplantation of tumors with a known response to ionizing radiation; (2) attachment of the cecum to the lateral abdominal wall with a permanent suture for the administration of ionizing radiation; and (3) transfection of cells with luciferase before tumor implantation for the assessment of the chemoradiotherapeutic interventions over time by bioluminescence imaging before the end of the study. This technique allows targeted delivery of ionizing radiation in an intraperitoneal tumor.

ers. For instance, p21 elevation in response to IR is an expected response of these radiosensitive cells. This was associated with an appropriate response of p53 leading to activation of p21 culminating in apoptosis as demonstrated by an elevation of the cleaved PARP-1. In HT29 cells, on the other hand, p53 was markedly elevated. This is the result of the mutated status of p53 in HT29 cells. However, the results with regards to Bax and survivin are not clear in these experiments as a decrease in survivin and Bax was expected in these radioresistant cells.

In separate *in vitro* studies, analysis with colorectal cancer cells with stable knock out (KO) of genes responsible for apoptosis from IR-induced injury was undertaken. This demonstrated that the p21 and the Bax KO genotypes were associated with radiosensitivity rather than radioresistance (Figure 6)[28]. The results with regards to p21 have been previously reported and indicate that it is mitotic catastrophe that leads these cells to undergo cellular death rather than becoming more radioresistant. The Bax KO genotype leading to a more radiosensitive phenotype as opposed to radioresistance was partly mediated by apoptosis inducing factor (AIF) and not to caspase mediated apoptosis[28]. AIF is an important mediator of cellular death that requires further studies as a predictor of a response to IR in rectal cancer[98].

These observations *in vitro* have been noted *in vivo* models of rectal cancer as well. However, one of the limitations of the studies *in vivo* is that these studies have relied on xenograft models of rectal cancer. We have previously described an orthotropic model in which cells have been implanted in the cecum and then the cecum was secured to the abdominal wall for targeted IR. Because these cells can be labeled with luciferase, the response to IR can be followed over time by biluminescence imaging (Figure 9). However, this model requires further validation[97].

In summary, observations from these studies demonstrate that there are good models for the study of rectal cancer in response to IR *in vitro* and *in vivo*. We have identified some molecules that can be used to predict a response to IR in HT-29 and HCT-116 cells. Application of these factors to the rest of the cells as depicted in Figure 3 has yielded mixed results. There is no unifying pathway that has been identified to date. Moreover, identification of predictors for a response to IR remain at large. For instance, many inhibitors of apoptosis examined (IAPs; survivin, XIAP, cIAP 1/2) were all increased in the more radiosensitive SW620 cells compared to the SW480 cells. Survivin, in response IR in colorectal cancer cells (0, 2, 4, and 6 Gy) was expressed in the following order in several cells: SW620 > HT-29 > HCT-116. Apoptosis was interrogated by PARP-1 cleavage and demonstrated that apoptosis in response to IR occurred in the following pattern: DLD-1 > HCT-116 > SW480 > HT-29 > SW480. p27 demonstrated the following pattern: HT-29 > HCT-116 > SW480. There was no particular pattern of expression of these factors nor was there a correlation to a response to IR noted. Thus, there is further need for identification of a unifying pathway that could be used to determine a response to IR.

The additional advantage of the current *in vitro* and *in vivo* models is that they can be utilized for the study of radiosensitizing agents and some of these have demonstrated promising results[92,94]. The effects of the radiosensitizing agents on specific pathways can also be explored in this fashion.

We then proceeded with a review of literature to determine how these observations compared to other studies. The result of this review have been previously documented to some extent and are presented and updated in the following discussion[22,99].

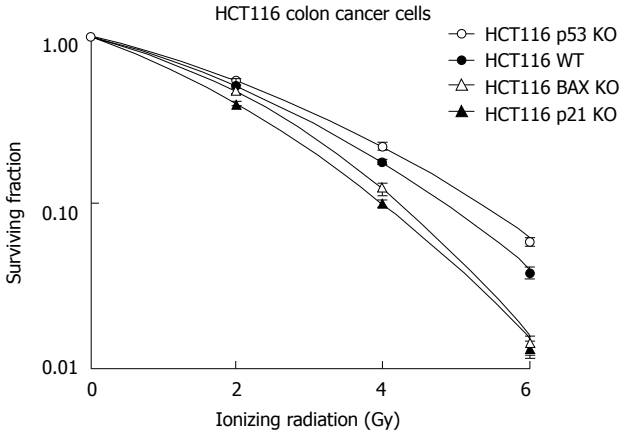

Figure 10 Analysis of HCT116 cells with stable KO genotypes for p53, p21, and Bax compared to wild-type. Cells deficient in p53 are more radioresistant, while p21 and Bax deficient cells are more radiosensitive compared to wild-type.

FACTORS THAT LEAD TO A RESPONSE TO IR: A REVIEW OF THE LITERATURE

Apoptosis

If cells are unable to repair the damage induced by IR, the cell is destined to undergo programmed cell death. In the classical pathway, the stressed cell leads to an up-regulation of p53, which then stops the cell cycle *via* induction of the cyclin depended kinase inhibitor p21. Failure to repair the damage causes BAX to induce apoptosis[22,100] (Figure 8).

It is conceivable that defects in any of these molecules (apoptotic or cell cycle proteins) alone or in combination could serve as a surrogate to predict a response to IR in rectal cancer. *In vitro* studies with colon cancer cells exposed to radiation have been in agreement with the classical response to apoptosis with p53, but not uniformly with p21 and BAX (as discussed in the previous section)[28] (Figure 8).

Apoptotic proteins: p53, p21, BAX, Bcl-2, survivin, and SMAC/Diablo

p53: *In vitro*, HCT-116 cells deficient of p53 are more radioresistant compared to HCT-116 wild-type cells. Tumor xenografts derived from the same cells demonstrated a similar effect[28]. These results have been mirrored in models of colorectal cancer *in vitro* and *in vivo*[101,102], but in disagreement with others[103-105]. Other studies have suggested that p53 mutations may render cells more radiosensitive owing to a reduction in p53-dependent DNA repair mechanisms[106]. Thus, *in vitro* and *in vivo* studies with regards to p53 have shown mixed results. *In vitro*, data indicates that lack of p53 leads to radioresistance. However, the mutational status of p53 is important to consider in all analyses examining p53[22].

Ex vivo studies have demonstrated a number of heterogeneous findings as well. Some studies have shown that mutated p53 leads to radioresistance in rectal cancer tissues[107]. Nuclear expression of p53 in rectal cancers predicted treatment failure and signified resistance to preoperative IR[96]. Other studies have demonstrated no usefulness of p53 as a marker of a response to IR[108,109]. To date, *ex vivo* studies have failed to provide usefulness as a marker of a response to IR. This might be the result of the low number of subjects included in the studies, the wide range of techniques utilized to detect p53, or the ability of the antibody to recognize the mutated *vs* the wild-type form of p53[22].

Cell cycle factors such as p53 and the cyclin dependent kinase inhibitors (CDKIs) (p21 and p27) have been studied as possible candidates to predict a response to ionizing radiation in rectal cancer. p21 is the classical CDKI and is activated by p53[110,111]. Irradiated colon cancer DLD-1 cells expressed low levels of p21[112]. The expected response to IR in cells and tumors deficient of p21 would be a radioresistant phenotype. Recent studies have shown that HCT-116 cell deficient of p21 are, in fact, more sensitive to ionizing radiation compared to wild-type HCT-116 cells[28,113]. Tumor xenografts deficient of p21 demonstrated more tumor regression compared to the wild-type genotype treated with the same dose of ionizing radiation[28].

p21: *Ex vivo* studies demonstrated the p21 positive tumors had a good response to IR[114]. Another study showed that p21 expression correlated with good pathological response and tumor radiosensitivity[115]. Similarly, a reduction by 50% in post-irradiated rectal tissue compared to pre-irradiated one was associated with radioresistance[116]. Another study did not find p21 useful as a predictor of a response to IR[117].

p27: This study found that p27 positive tumors had a better response to IR with an OR of 3.3[117]. Similarly, the absence of p53 and p27 prior to treatment was associated with poor response to IR in rectal tumors[118].

Bax: Bax is a pro-apoptotic protein that leads to the release of cytochrome c from the intermitochondrial membrane[100]. It may be anticipated that Bax deficiency would be associated with radioresistance. *In vitro* and *in vivo* studies have demonstrated the opposite phenotype to IR (Figure 10)[28]. While a few studies demonstrate that Bax deficient cells are resistant to chemotherapeutic agents[119-121], evidence indicating the response of Bax deficient colorectal cancer cells to IR in pre-clinical studies is lacking. Limited *ex vivo* studies have shown that Bax tumor expression had a positive response to chemoradiation in patients treated for rectal cancer[122,123].

Bcl-2 inhibits cellular apoptosis and is overexpressed in many colorectal tumors[124]. BAX is the apoptogenic counter part of Bcl-2. Current studies have failed to demonstrate the association of Bcl-2 as a marker of response to IR[22,123,125].

Survivin: Survivin is one of eight inhibitors of apopto-

sis (IAPs) that are generated *via* induction of NFκB[100]. Survivin binds and inactivates caspases 3, 7 and 9[100]. *In vitro* and *in vivo* data showed that the NFκB-IAPs axis is a predictor of a poor response to IR when over expressed[22]. *Ex vivo* data supports the role of survivin in raidoresistance[93]. Furthermore, the five year survival of patients with survivin positive stage Ⅱ colon cancer tumors was 41% lower than patients with survivin negative tumors[126]. The role of other r IAPs (*i.e.*, XIAP, cIAP, *etc.*) and a response to IR remains at large.

The role of the IAPs in response to IR has been further interrogated by directly inhibiting the inhibition of the IAPs *via* augmentation of an antagonistic factor to the IAPs: SMAC/Diablo.

SMAC/Diablo: Pro-apoptotic molecules with the ability to reduce the functional activity of the inhibitors of apoptosis might have potential therapeutic applications. Compounds that mimic the action of SMAC/Diablo (Smac-mimetics) are under study for their ability to chemo- and radiosensitize tumor cells[127]. The Smac mimetic JP-1201 radiosensitized HT-29 colorectal cancer cells and xenografts by a marked augmentation in apoptosis, which was associated with a reduction in the levels of the IAP XIAP[94].

Proliferation markers and mitotic index as markers: A few studies have reported high Ki-67 staining correlated with a positive response to IR[128,129]. In contrast, most studies have demonstrated that proliferating nuclear antigen labeling index does not correlate with response to IR[115,125,130].

Apoptotic index: Evaluation of apoptosis in cancer cells has shown that patients with higher pre-radiation level of apoptosis (apoptotic index) had lower rate of recurrence and longer disease free period after radiation[131].

Logically, tumors that have an intact machinery to undergo apoptosis should respond better to ionizing radiation rather that those with mutation of one or more pro-apoptotic factors or activation of anti-apoptotic factors. Caspase mediated apoptosis has been shown to play a promising role in predicting a response to IR. A high spontaneous apoptotic index in pretreated tumor tissue was associated with a superior rate of response to radiation[132]. Furthermore, in a large study including 465 pre-irradiated biopsies tumors underwent immunohistochemistry staining against the active form of caspase 3. This study showed that tumors with a high apoptotic index had less recurrence and a higher disease free survival[131].

While these results seem promising, uniformity across studies has not been established nor substantial reproducibility or adoption to clinical practice. The practical usefulness of this approach is limited by the dynamic process of apoptosis and by the wide variety of measurements and laboratory standardizations. The individual evaluation of specific molecules in the process of apoptosis either as a single factor or in combination with others seems to suffer from the same issues.

Hypoxia and angiogenic factors

Hypoxia: Lack of oxygen supply to cancer cells has been linked to poor response to radiation. This premise was tested in patients undergoing neoadjuvant therapy for rectal cancer with the assistance of positron emission tomography using the copper-60-diacetyl-bis (N4-methylthiosemicarbazone (^{60}Cu-ATSM), an agent that accumulates in tissues lacking adequate oxygenation. Tumors with higher baseline tumor-muscle activity ratios (suggesting hypoxia) in the pre-treatment PET scan were shown to have a poor response to radiation[133]. Other agents tested in different studies have been less useful probably as a result of technical limitations[134].

Further evidence of the role of hypoxia in response to IR was demonstrated by the fact that higher levels of HIF-1 (hypoxia inducible protein factor 1, a protein that increases in oxygen deprived tissues) predicts poor response to neoadjuvant chemotherapy in patients with rectal cancer[135]. Additionally, HIF-1 correlates with increased levels of pro-angiogenic vascular endothelial growth factor (VEGF), a marker of angiogenesis for tumor growth[136].

VEGF: Low levels of VEGF have been associated with improved response to radiation[135,137,138], and vice versa[135,137-139]. Therefore, VEGF inhibition with the antibody bevacizumab has shown beneficial effects in treating cancers with neoadjuvant chemoradiotherapy[137,140,141]. Various mechanisms by which VEGF inhibition causes this effect may include reducing vascular density within a tumor, decreasing interstitial tumor pressures, improving global oxygenation status, vascular normalization and thus increasing responsiveness of endothelial cells to radiation[137,141,142]. It seems logical that if bevacizumab were to be used as a neoadjuvant agent in combination with IR for the treatment of patients with rectal cancer, these should have a higher rate of pCR compared to standard treatments. However, this observation has not been validated in clinical trials[143].

EGFR signaling: Initial reports revealed that combination of radiation and EGFR inhibition exerted a synergistic cytotoxic effect and hence raised interest in developing EGFR inhibitors. Hence, multiple EGFR inhibitors (*e.g.*, cetuximab and panitumumab) were developed and tested and have demonstrated promise in patients with KRAS wild-type tumors. However, with regards to the usefulness in EGFR signaling as a predictor of a response to IR, the data is lacking. Similarly, data pertaining to the usefulness of inhibiting the EGFR signaling pathway as a radiosensitizing modality has also demonstrated disappointing results[144].

High-throughput analyses

Microarray analysis: Single molecules as independent factors or in combination with other molecules of specific pathways (*i.e.*, apoptosis or angiogenesis) have not provided to be clinically useful to date. A major limita-

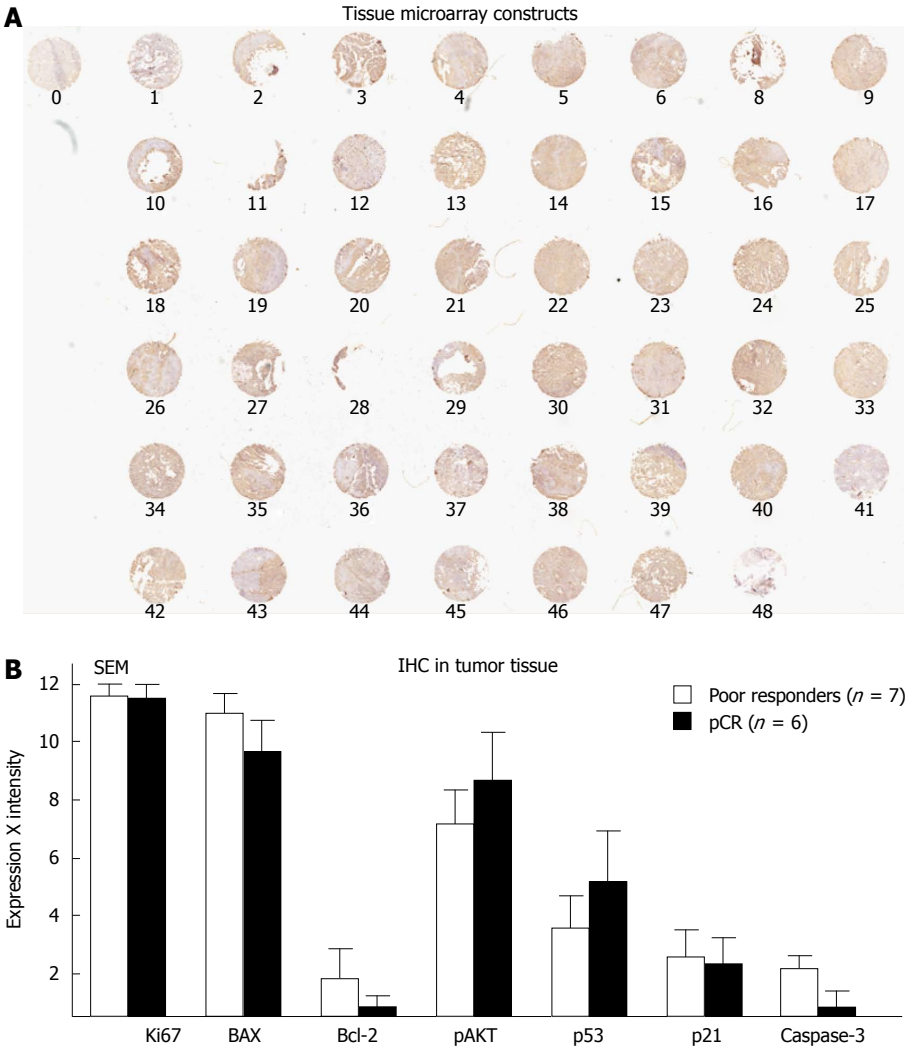

Figure 11 Tissue Microarray Constructs were created with 48 patients with rectal cancer that received pre-operative radiation. A: Of these 48 patients (each dot represents a patient), six had a pCR and seven did not respond to treatment; B: The differences in various tumor markers comparing these two groups. IHC: Immunohistochemistry; SEM: Scanning electron microscope.

tion of examining a specific pathway had to do with the dynamics of the process and the particular point in time at which it is being measured. Further, many tumors are heterogeneous in terms of mutations and alterations. Thus, interrogating several genes or proteins simultaneously is a logical approach in terms of elucidating origins of radioresistance in rectal cancer. In the era of personalized care, these tumor "fingerprints" not only make sense, but is the direction of the future.

Unfortunately, as appealing as it might seem, current efforts have been unsuccessful. Two studies have independently performed RNA arrays to analyze radioresistant and radiosensitive tumors. These studies have had limited genes and have had different results[145,146].

Tissue microarray: Tissue microarray is another technique to assess multiple proteins with a single experiment with tissues handled in a similar fashion. In one study, tissue microarray was performed with the goal of predicting survival and recurrence in patients treated with chemoradiation. In this study, Cox-2 emerged as a potential predictor of survival[147]. In a second study, our group subjected rectal cancer tissue to tissue microarray and tested eight different antibodies. MIB was the only independent predictor of a response to chemoradiation[8]. In our analysis, we examined tissue microarray in 48 patients who were treated with preoperative IR. We then divided all of these patients in two groups: patients who achieved a pCR (n = 6) compared to those who did not respond to IR or patients who experienced tumor growth (n = 7) in spite of pre-operative chemoradiation. We stained the tissue microarrays with seven antibodies and demonstrated no particular protein that could be used to differentiate these groups (Figure 11)[8].

CONCLUSION

Rectal cancer is the ideal clinical problem where personalized treatment could be investigated. This theory stems from the fact that a select patient population obtains an excellent response from the same form of chemoradiation, while others do not. Despite putting forward multiple mechanisms of tumor death from ionizing radiation and various possible causes of radioresistance, there

has not been a unifying pathway that can reliably predict a response to IR *in vitro*, *in vivo* or *ex vivo*. It is difficult to explain the reasons behind a clear discrepancy in the current observations in the literature. However, differences in tumor biology, genotypic profiling or phenotypic characteristics are some of these factors. There are currently good *in vitro*, *in vivo*, and *ex vivo* models for the study of rectal cancer and the trend seems optimistic in developing a predictive finger print for patients with rectal cancer that might respond well to IR. Recent data has shown that DNA-PKcs and Ku proteins (as vital players in NHEJ pathway allowing DSB repair) may have a central role in radiation induced cell death. Nevertheless many facets of its function in conjunction with the complex and intricate details of the pathway are still under investigation. More data is required before we can formulate one unified explanation for the heterogeneity noted in therapeutic effect of ionizing radiation. Until then, the hope of developing novel therapies for rectal cancer and improving the therapeutic yield of ionizing radiation with radiosensitizers remains a challenging clinical problem. The findings so far should not be viewed in a pessimistic fashion. There are several pathways that have provided potential targets for chemoradiotherapeutic interventions. We need to continue to investigate potential molecules predictive of a response to IR. As we dwell into the future, we need to remember that markers predictive of an aggressive behavior are currently in clinical practice such as testing for BRCA or RET proto-oncogene mutations. A view into the future also includes investigating base line characteristics of patient's genotypic background in normal tissue compared to tumor tissue after IR. It is important to determine if a patient starts with high levels at base line, but a particular gene is not activated then the base line levels are not as predictive. In the opposite scenario, we might have a patient with a molecule that at base line is low, but it is activated substantially with IR. In that scenario, we might consider those features as more predictive. The future, therefore, should be viewed with optimism.

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REVIEW

Advances and new perspectives in the treatment of metastatic colon cancer

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Abstract

During the last decade we have witnessed an unprecedented outburst of new treatment approaches for the management of metastatic colon cancer. Anti-angiogenic drugs, epidermal growth factor receptor blockers and multi-kinase inhibitors have all resulted in small but consistent improvement in clinical outcomes. However, this progress has paradoxically leaded us into new challenges. In many cases the clinical development was done in parallel and the lack of head-to-head comparison evolved into circumstances where several valid new "standards of care" are available. Even though desirable in essence, the availability of many options as well as different possible combinations frequently leaves the busy clinician in the difficult situation of having to choose between one or the other, sometimes without solid evidence to support each decision. In addition, progress never stops and new agents are continuously tested. For these reason this review will try to summarize all the clinical trials that constitute the theoretical framework that support our daily practice but will also procure the reader with rational answers to common clinical dilemmas by critically appraising the current literature. Lastly, we will provide with a compilation of promising new agents that may soon become our next line of defense against this deadly disease.

Key words: Colon Cancer; Stage Ⅳ; Metastatic; Review; Bevacizumab; Cetuximab; Panitumumab; Aflibercept; Regorafenib

Core tip: This manuscript is a comprehensive review, with the most updated information up to 2014, regarding metastatic colon cancer. It summarizes all those relevant clinical trials that constitute the theoretical framework to support our daily practice and provides rational answers to common clinical dilemmas. Additionally, it gives the reader a compilation of potential new agents that are currently being tested and may soon become the next step in the battle against this disease.

Recondo G Jr, Díaz-Cantón E, de la Vega M, Greco M, Recondo G Sr, Valsecchi ME. Advances and new perspectives in the treatment of metastatic colon cancer. *World J Gastrointest Oncol* 2014; 6(7): 211-224 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i7/211.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i7.211

INTRODUCTION

Colon cancer is the second leading cause of cancer-related mortality in the United States and 1.2 millions of

new cases are yearly diagnosed worldwide[1]. From the clinical perspective colon cancer could be categorized into the early stages (Ⅰ-Ⅲ) and the more advanced and usually lethal metastatic disease. Notably, since the publication of the MOSAIC trial almost ten years ago, no other groundbreaking development in the treatment of resectable colon cancer became available[2]. On the contrary, during the last decade we have witnessed an unprecedented outburst of new treatment approaches for the management of stage Ⅳ colon cancer that ultimately evolved into the approval of five new drugs. For simplification purposes, we can subdivide these new drugs into three categories: anti-angiogenic, epidermal growth factor receptor (EGFR) blockers and multi-kinase inhibitors. All of them represent important advances in the fight against this deadly disease. Nonetheless, some issues deserve further attention. First, these new agents were generally combined with at least some of the previously effective chemotherapy regimens (fluoropyrimidines and/or oxaliplatin and/or irinotecan). Also, the clinical development was done in parallel instead of following a rational stepwise approach where each new drug was tested against the new standard of care. This lack of head-to-head comparison resulted in several valid new "standards of care". Lastly, new combinations are continuously tested making extremely difficult for the busy clinician to keep up with the most updated information.

For the reasons mentioned before, this manuscript will pursue three clear objectives. First summarize all those relevant clinical trials that constitute the theoretical framework to support our daily practice. Second try to provide rational answers to common clinical dilemmas by critically appraising the current literature. Finally, provide the reader with a compilation of potential new agents that are currently being tested and may soon become the next step in the battle against this disease.

ANTI-ANGIOGENESIS AS A TARGET

Angiogenesis consists in a complex multistep process of new vessel formation. The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play a crucial role in the tumor transition from the "avascular" to the "vascular" phase, acquiring metastatic potential[3,4]. It also stimulates tumor growth, migration and metastasis through mechanisms not entirely related to tumor angiogenesis[5]. Moreover, tissue interstitial pressure is a key factor in chemotherapy delivery and in some tumors this could be up to 15 times higher than the normal counterparts[6]. There is solid evidence that VEGFR inhibition partially restores interstitial fluid pressure and reduces abnormal vasculature with improvement of drug delivery and enhancement of chemotherapy efficacy[7].

Bevacizumab

Bevacizumab (Avastin®, Genentech Inc.), a recombinant humanized monoclonal IgG-1 antibody against soluble VEGF-A, was the first anti-angiogenic drug approved for metastatic colon cancer. It prevents the binding of VEGF-A to the VEGFR and, consequently, inhibits angiogenesis, tumor growth and metastatic development. It was first approved on February 2004 by the FDA as first-line treatment for patients with metastatic colon cancer. Today, almost 10 years later, a substantial body of evidence has accumulated to help clinicians in the judicious use of this molecule. Table 1 summarizes the most relevant clinical trial of the anti-angiogenic drugs.

The first practice-changing, double blind, randomized phase Ⅲ trial that was published compared the use of irinotecan, bolus 5-FU and leucovorin (IFL) with or without bevacizumab in metastatic, previously untreated patients[8]. The primary endpoint of the study was overall survival (OS); disease-free survival (DFS) and overall response rate (ORR) were secondary endpoints. OS (20.3 mo *vs* 15.6 mo; $P < 0.001$) and PFS (10.6 mo *vs* 6.2 mo; $P < 0.001$) and ORR (45% *vs* 35%) were all significantly improved with bevacizumab. Importantly, patients in the IFL group were not allowed to crossover. Similar results were obtained in the ARTIST trial using a modified version of IFL (5-FU was infused over 6-8 h) plus bevacizumab in metastatic colon cancer, chemotherapy naïve, Chinese patients, confirming that results obtained in Caucasians were also applicable in Asian population[9]. Subsequently, in 2007 results from the BICC-C trial were released showing that bevacizumab combined with the classical bolus and 46-h infusional 5-FU plus leucovorin and irinotecan (FOLFIRI) was superior to a shorter version of IFL as upfront therapy[10]. In the original trial design patients were randomly assigned to receive FOLFIRI, IFL or irinotecan plus capecitabine (CapeIRI) with or without celecoxib. However, after the FDA-approval of bevacizumab the protocol was amended and additional 117 patients were randomized to receive bevacizumab with FOLFIRI (FOLFIRI-B) or IFL (IFL-B); due to excessive toxicity the CapeIRI arm was discontinued. With an updated median follow-up of 34.4 mo, OS was longer in the FOLFIRI-B arm (28.0 mo *vs* 19.2 mo; $P = 0.037$)[11]. Thus, infusional 5-FU regimens should be preferred over bolus 5-FU when combined with bevacizumab.

After the initial success with irinotecan combinations, bevacizumab was soon studied in oxaliplatin-based regimens. The first evidence of its synergistic effect came from the ECOG-3200 study that investigated the role of bevacizumab in the second line treatment[12]. In this study patients who had progressed to irinotecan and fluoropyrimidine therapies but who had not received oxaliplatin or bevacizumab were randomized to FOLFOX-4 (control arm), FOLFOX-4 plus bevacizumab (FOLFOX-B) or single agent bevacizumab. With a median follow-up of 28-mo, a modest but statistically significant improvement in OS was shown for the FOLFOX-B arm (12.9 mo *vs* 10.8 mo, $P = 0.0024$). Single agent bevacizumab showed virtually no effect. Immediately after the release of this study, and in spite of the lack of evidence in the front line therapy setting, FOLFOX-B was rapidly accepted in the oncology community as a valid front line option for stage Ⅳ colon cancer. Evidence to support this practice finally materialized in 2008. The NO16966 study was a

Table 1 Selected phase 3 clinical trials involving anti-angiogenic drugs in combination with conventional chemotherapy

Ref.	Drug and study name	Study description	No. of patients	Comparison	Median OS (mo)	Median TTP/PFS (mo)	ORR	1-yr survival
	Bevacizumab (B)							
Hurwitz *et al*[8] 2004	AVF2107g trial	RCT, 1st line	813 (ITT)	IFL + B *vs* IFL	20.3 *vs* 15.6	10.6 *vs* 6.2	45% *vs* 35%	74% *vs* 63%
Fuchs *et al*[10] 2007	BICC-C trial	RCT, 1st line	117 (2nd period)	FOLFIRI + B *vs* mIFL + B	28 *vs* 19	11 *vs* 8	58% *vs* 53%	87% *vs* 61%
Giantonio *et al*[12] 2007	ECOG 3200 trial	RCT, 2nd line post irinotecan 1st line	820 (ITT)	FOLFOX-4 + B *vs* FOLFOX-4 *vs* B alone	12.9 *vs* 10.8 *vs* 10.2	7.3 *vs* 4.7 *vs* 2.7	23% *vs* 8.6% *vs* 3.3%	56% *vs* 43% *vs* 44%
Saltz *et al*[13] 2008	NO16966 trial	RCT, phase 3, 1st line, factorial 2 x 2	1401	FOLFOX-4 or XELOX + B *vs* FOLFOX-4 or XELOX	21.3 *vs* 19.9	9.4 *vs* 8.0	47% *vs* 49%	Not reported
Tebbutt *et al*[17] 2010	MAX trial	RCT, open label, 1st line	471	Cape alone *vs* Cape + B *vs* Cape + B + mitomycin	18.9 *vs* 18.9 *vs* 16.4	5.7 *vs* 8.5 *vs* 8.4	30% *vs* 38% *vs* 46%	Not reported
Cunningham *et al*[18] 2013	AVEX trial	RCT, elder population, 1st line	280	Cape alone *vs* Cape + B	20.7 *vs* 16.8	9.1 *vs* 5.1	19% *vs* 10%	74% *vs* 44%
Falcone *et al*[21] 2013	TRIBE trial	RCT, 1st line	508	FOLFOXIRI-B *vs* FOLFIRI-B	31.0 *vs* 25.8	12.1 *vs* 9.7	65% *vs* 53%	Not reported
Bennouna *et al*[66] 2013	ML 18147	RCT, open label, 2nd line post chemo + B	409	2nd line chemotherapy + B *vs* 2nd line chemotherapy	11.2 *vs* 9.8	5.7 *vs* 4.1	5.5% *vs* 4%	Not reported (approximately 50% *vs* 40%)
	Ziv-Aflibercept							
Van Cutsem *et al*[29] 2012	VELOUR trial	RCT, 2nd line post oxaliplatin and/or bevacizumab 1st line	1226	FOLFIRI + aflibercept *vs* FOLFIRI + placebo	13.5 *vs* 12.0	6.9 *vs* 4.7	20% *vs* 11%	56% *vs* 50%

RCT: Randomized controlled trial; OS: Overall survival; TTP: Time to progression; PFS: Progression free survival; ITT: Intention to treat; ORR: Overall response rate.

non-inferiority trial evaluating the use of XELOX and FOLFOX with or without bevacizumab in a factorial design[13]. The primary analysis demonstrated a statistically significant benefit in terms of progression-free survival (PFS) (9.4 mo *vs* 8.0 mo; $P = 0.002$) in patients receiving bevacizumab, irrespectively of the chemotherapy backbone used, but there was no difference in terms of OS and ORR in the final analysis. Moreover, the TREE studies evaluated the use of three different oxaliplatin-based chemotherapies with bevacizumab[14]. A total of 150 patients were randomly assigned to mFOLFOX-6, bFOL (bolus FU and low-dose LV with oxaliplatin) or CapeOx in the TREE-1 cohort and 223 patients were randomized to the same regimens with bevacizumab in the TREE-2 cohort. ORR was superior in each arm with the addition of bevacizumab and, although not statistically significant, it was highest with mFOLFOX-6 and bevacizumab (52%). Additionally, the BEAT study was designed to evaluate the safety and efficacy of several regimens containing bevacizumab used in the daily community practice but outside the formalities of a clinical trial and in a no-comparative fashion[15]. Consistent with previous studies, improved PFS and OS were seen in patients receiving doublet regimens compared to single agent chemotherapy.

A very relevant issue, however, for the daily practice is the fact that many patients with metastatic colon cancer are not suitable (*e.g.*, elder population or poor performance status) to receive multi-agents regimen such as FOLFOX or FOLFIRI. A common practice in these cases is to use single agent fluoropyrimidine (*e.g.*, weekly bolus 5-FU). Even in this situation, there is enough evidence to support the use of bevacizumab. At least one phase II clinical trial proved that the addition of bevacizumab to single agent 5-FU resulted in better PFS (9.2 mo *vs* 5.5 mo, $P < 0.001$) when used as first line option[16]. Importantly, the mean age of the participants was more than 70 years old. Further evidence supporting the efficacy of this combination, especially in fragile patients, came from the MAX study where capecitabine and bevacizumab resulted in longer PFS compared to single agent capecitabine (8.5 mo *vs* 5.7 mo; $P < 0.001$)[17]. This was confirmed by the AVEX Trial that enrolled elder patients (> 70 years) who were not candidates for treatment with oxaliplatin or irinotecan and randomized them to capecitabine alone or in combination with bevacizumab[18]. With a mean follow up close to 2 years, the median PFS was almost double with bevacizumab (9.1 mo *vs* 5.1 mo; $P < 0.001$). ORR was also superior but the study was underpowered to detect a benefit in OS. However, the reader should be aware that the addition of bevacizumab in these three trials resulted in an absolute increment of grade 3-4 toxicity of about 15%-20% with none of them showing a statistically benefit in OS.

A classical paradigm that has been recently called into challenge is the one that discourage the use of multi-agents regimens combining oxaliplatin and irinotecan at the same

time. This presumption was based on the results of the N9741 study where the IROX (oxaliplatin + irinotecan) arm showed worse TTP, ORR and OS compare to FOLFOX[19]. However, treatment with the combination of 48-h infusional 5-FU, oxaliplatin and irinotecan (FOLFOXIRI) proved to be superior to FOLFIRI, which is believed to be similar to FOLFOX, in terms of OS, PFS and ORR in patients with mCC[20]. Recently, the results of a phase 3 TRIBE trial that compared FOLFOXIRI and FOLFIRI with the addition of bevacizumab were presented[21]. Both treatments were administered for a maximum of 12 cycles followed by 5-FU + bevacizumab until progression. With a mean follow-up of 26.6 mo, significantly increased PFS was observed in the FOLFOXIRI-B arm (9.7 mo *vs* 12.2 mo, $P = 0.001$). As expected, greater neutropenia, diarrhea, stomatitis and neurotoxicity were seen in the FOLFOXIRI arm. Interesting, similar results were obtained in a recent randomized phase Ⅱ study (OLIVIA) where FOLFOXIRI-B showed better ORR and conversion to R0 resections compared to FOLFOX-B[22]. Data is still immature, but this combination could be a feasible option for fit patients.

To summarize we should emphasize some useful concepts. First, single agent bevacizumab has almost no activity. Second, the best evidence comes from its usage as upfront first line therapy in combination with either FOLFOX or FOLFIRI and perhaps FOLFOXIRI. In all cases, bevacizumab has persistently showed to improve PFS. For second line treatment the ideal scenario would be in patient who did not receive bevacizumab as a first line option. Lastly, continuation beyond progression is also feasible (see below).

Ziv-aflibercept

Ziv-aflibercept (Zaltrap®, Regeneron Pharmaceuticals) is a recombinant fusion protein consisting of the extracellular domains of human VEGFR-1 and 2 fused to the Fc portion of human IgG-1[23]. The decoy protein binds tightly PIGF, VEGF-A and VEGF-B preventing the activation of VEGFR-1 and 2 by these ligands. This is a significant difference with bevacizumab which exclusively blocks the VEGF-A[24]. Pre-clinical studies confirmed that when combined with cytotoxic drugs, ziv-aflibercept exerted considerable inhibition of angiogenesis[25-27]. In 2006, 38 patients were enrolled in a phase I clinical trial were 2, 4, 5 and 6 mg/kg escalating doses of ziv-aflibercept were explored in combination with irinotecan, 5-FU and leucovorin[28]. In the phase 3 VELOUR trial, patients with metastatic colon cancer but previously treated with oxaliplatin-containing regimens were randomly assigned to receive FOLFIRI with or without ziv-aflibercept every 2 wk[29]. Patients could not have received irinotecan before but up to 30% of them received bevacizumab as front line therapy. The ORR (11.1% *vs* 19.8%, $P < 0.001$), PFS (6.9 mo *vs* 4.6 mo, $P < 0.001$) and OS (13.5 mo *vs* 12.1 mo, $P = 0.003$) were all improved in ziv-aflibercept and were not influenced by the prior use of bevacizumab (stratifying variable). However, the absolute benefit was a modest 1.4 mo in OS.

BLOCKING EGFR AND OTHER KINASES

Cetuximab and panitumumab

In addition of blocking the angiogenesis pathway, another line of investigation that lead to practice-changing outcomes was the one advocated to jamming the EGFR. Once activated, the EGFR triggers a series of downstream phenomenon that ultimately result in tumor growth and survival[30]. It is then simple to understand that blocking EGFR could potentially halt tumor progression. Nevertheless, this basic principle is not always applicable. An overwhelming body of evidence confirmed the futility of blocking the EGFR when downstream molecules are anarchically activated. The strongest evidence comes from the presence of KRAS codons 12 and 13 mutations in exon 2 which virtually turns anti-EGFR strategies useless[31]. But, recent investigations have broadened the number of negative predictive mutations found in the RAS genes family to exons 3 and 4 of KRAS and exons 2, 3 and 4 of NRAS genes[32]. In that sense, testing for KRAS/NRAS mutations could exclude 50% of the patients from an ineffective but potentially harmful therapy. BRAF mutations carry a considerable poor prognosis, but its predictive role is somehow controversial. However, and in spite of this obvious limitation, anti-EGFR therapies have found their place in the treatment of stage Ⅳ colon cancer. Two compounds, cetuximab (Erbitux®, Bristol-Myers) a chimeric monoclonal IgG-1 antibody against EGFR, and panitumumab (Vertibix®, Amgen) a fully humanized monoclonal IgG-2 antibody also directed against EGFR, have received FDA-approval for this indication. Table 2 summarizes the most relevant clinical trials related to these agents.

As part of the pre-clinical investigation, cetuximab was tested in tumor xenografts models and found to have marked synergistic activity with irinotecan, even in previously considered irinotecan-resistant cell lines[33]. This observation was the based for a couple of phase 2 clinical trials which confirmed the clinical utility of cetuximab single agent (approximately 10% ORR) and in combination with irinotecan. However, the first convincing evidence of its clinical utility came from the BOND study where 329 patients with irinotecan-resistant metastatic colon cancer were randomly assigned to either single agent cetuximab (ORR 11%, TTP 1.5 mo) or cetuximab plus irinotecan (ORR 23%, TTP 4.1 mo)[34]. No difference in OS was seen but crossover was allowed. As in the case of cetuximab, single agent panitumumab showed 10% ORR in heavily pretreated patients who formerly received 5-FU, irinotecan and/or oxaliplatin[35,36]. Given the encouraging results as second and third line therapies, it did not take much time until both molecules were tested as first line options. In the CRYSTAL trial, 1217 patients were randomly assigned to FOLFIRI alone or FOLFIRI plus cetuximab as first line treatment[37]. The primary endpoint was PFS and it was statistically prolonged in the cetuximab group, albeit by a modest 1 mo (8.0 mo *vs* 8.9 mo in the whole population and 8.7 mo *vs* 9.9 mo in the KRAS wild-type patients). Cetuximab also resulted in

Table 2 Selected clinical trials involving anti-epidermal growth factor receptor, regorafenib or anti-epidermal growth factor receptor *vs* anti-epidermal growth factor receptor receptor agents

Ref.	Drug and study name	Study description	No. of patients	Comparison	Median OS (mo)	Median TTP/ PFS (mo)	ORR	1-yr survival
	Cetuximab (*C*)							
Cunningham *et al*[34] 2004	BOND trial	RCT, phase 2, 2nd line irinotecan-refractory	329	Irinotecan + C *vs* irinotecan	8.6 *vs* 6.9	4.1 *vs* 1.5	23% *vs* 11%	29% *vs* 32%
Van Cutsem *et al*[37] 2009	CRYSTAL trial	RCT, 1st line	1198	FOLFIRI + C *vs* FOLFIRI	20 *vs* 18.5 and (25 *vs* 21)	9 *vs* 8 and (10 *vs* 8.7)	47% *vs* 39% (59 *vs* 43%)	Not reported (approximately 35% *vs* 25%)
Maughan *et al*[59] 2011	COIN trial	RCT, phase 3, 1st line	729 (KRAS wild type)	Oxaliplatin-based chemo + C *vs* chemo alone	17 *vs* 17.9	8.6 *vs* 8.6	64% *vs* 57%	Not reported
Tveit *et al*[60] 2011	NORDIC VII trial	RCT, open label, 1st line	571	FLOX + C *vs* intermittent FLOX + C *vs* FLOX	19.7 *vs* 20.3 *vs* 20.4	8.3 *vs* 7.3 *vs* 7.9	49% *vs* 47% *vs* 41%	Not reported (approximately 70%)
	Panitumumab (*P*)							
Douillard *et al*[39] 2010	PRIME trial	RCT, phase 3, 1st line	1183	FOLFOX-4 + P *vs* FOLFOX-4	24 *vs* 20 (WT) 15 *vs* 19 (MT)	9.6 *vs* 8 (WT) 7.3 *vs* 8.8 (MT)	55 *vs* 48% (WT) 40 *vs* 40% (MT)	Approximately 75% both (WT) approximately 60% *vs* 75% (MT)
	Regorafenib (*R*)							
Grothey *et al*[47] 2013	CORRECT trial	RCT, phase 3, 3rd line	760	Regorafenib *vs* placebo	6.4 *vs* 5.0	1.9 *vs* 1.7	1.0% *vs* 0.4%	24.3% *vs* 20.0%
	Cetuximab (*C*) *vs Bevacizumab* (*B*)							
Stintzing *et al*[63] 2013	FIRE-3trial	RCT, phase 3, 1st line	592	FOLFIRI + C *vs* FOLFIRI + B	28.7 *vs* 25	10 *vs* 10.3	62 % *vs* 58%	Not reported

RCT: Randomized controlled trial; OS: Overall survival; TTP: Time to progression; PFS: Progression free survival; ITT: Intention to treat; ORR: Overall response rate.

an absolute 8% improvement in ORR (all partial responses) but no benefit in OS was observed. Similar results were reported in a randomized, phase 2 study using FOLFOX instead of FOLFIRI[38]. In this case the ORR was improved by 25% in wild-type patients as it was PFS, but only by 15 d (7.2 mo *vs* 7.7 mo). Interesting, in KRAS mutated patients PFS was actually 3-mo worse in the cetuximab arm. Similarly, in the phase 3 PRIME study, investigators used FOLFOX-4 as the backbone to randomized patients in a 1:1 fashion to panitumumab or placebo[39]. As expected, in the wild-type population ORR (48% *vs* 55%) and PFS (8.0 mo *vs* 9.6 mo) was better with anti-EGFR therapy but in KRAS mutated cases the effect was neutral or even worse.

An important point to mention at this moment is in reference to the solid evidence against the presumption that combining both anti-angiogenic and anti-EGFR molecules at the same time would results in a synergistic effect. At least two large, randomized, phase 3 clinical trials consistently showed that combining bevacizumab with EGFR inhibitors is actually deleterious. The first of them (PACCE trial) randomly assigned 1053 patients to either oxaliplatin- or irinotecan-based chemotherapy plus bevacizumab but with and without panitumumab as first line treatment for metastatic colon cancer[40]. The primary objective for the oxaliplatin-based arm was extension of PFS and in the irinotecan group was safety analysis. Secondary end points for both groups were ORR, OS and safety. A planned interim analysis for safety and efficacy was conducted at 50% of the events and panitumumab was removed due to significantly decreased PFS [hazzd ratio (HR), 1.44; $P = 0.004$] and increase toxicity independently of the KRAS status. Grade 3 or more adverse events were present in 90% of patients treated with panitumumab. The CAIRO-2 trial reported similar detrimental results of adding cetuximab to oxaliplatin, capecitabine and bevacizumab[41]. The addition of cetuximab significantly decreased median PFS (10.7 *vs* 9.4, $P = 0.01$). A total of 88% of patients discontinued the study, 45% due to tumor progression and 24.5% due to adverse events. A third study, the CALGB 80405, was initially designed to evaluate the use of FOLFOX or FOLFIRI with bevacizumab, cetuximab, or both agents together. In base of the results of the previous studies, the arm combining cetuximab and bevacizumab was closed (NCT00265850).

Regorafenib

The last drug to receive FDA-approval was regorafenib (Stivarga®, Bayer). The compound is an orally available multi-kinase inhibitor with activity against multiple targets in-

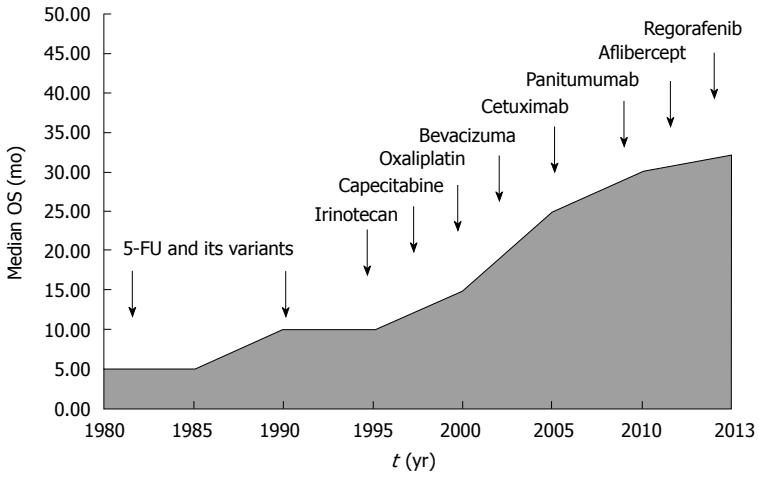

Figure 1 Schematic representation of the recent advances in the treatment of metastatic colon cancer.

cluding KIT, PDGFR and VEGFR among others. It is structurally related to sorafenib and the most usual adverse events are hand-foot skin reaction, mucositis, hypertension and diarrhea[42-45]. In an expanded phase I trial with 27 evaluable patients, 74% achieved disease control with 1 patient obtaining partial response and 19 stable disease[46]. Globally, regorafenib was well tolerated and adverse events were clinically manageable leading to a multi-centric phase 3 trial. The CORRECT study enrolled patients who had already received all the approved standard therapies and who had progressed during or within 3 mo after the last therapy[47]. Seven hundred and sixty participants were randomized in a 2:1 ratio to regorafenib or placebo. Median OS was 6.4 mo in the regorafenib group *vs* 5.0 mo in the placebo group (P = 0.005). The most frequent grade 3 or 4 adverse events were hand-foot skin reaction (17%), fatigue (10%), diarrhea (7%), hypertension (7%), and skin desquamation (6%).

COMMON CLINICAL DILEMMAS

We have witnessed an exponential growth in the number of clinical trials dedicated to metastatic colon cancer which eventually resulted in small but consistent improvement in clinical outcomes (Figure 1). However, this progress has paradoxically leaded us into new challenges. We have arbitrarily chosen 3 topics that in our own opinion are probably the more relevant clinical dilemmas. The reader should be aware, though, that the opinions expressed below come from our own assessment of the literature and they should be considered only as the authors' point of view.

Is there any role for peri-operative chemotherapy in potentially resectable liver metastases? Can the new biological agents improve the resectability rate on patients with borderline or unresectable liver metastases? Which regimen to chose?

The first point to consider is whether the patient has upfront resectable disease or not. A set of criteria have been proposed, however in any case this decision require appropriate discussion between the medical and surgical oncologists[48]. For those who are considered resectable common practice is to give them at least 6 mo of chemotherapy. The most solid evidence for this action comes from the EORTC 40983 trial where 364 patients, with one to four resectable liver metastases, were randomly assigned to surgery alone or 6 doses of FOLFOX-4 pre- and post-surgery[49]. The study was positive for its primary endpoint, PFS (20.9 *vs* 12.5; P = 0.035, per protocol population) and it gained rapid acceptance within the medical community. Oncologist extrapolated these results to the completely neo-adjuvant or adjuvant (stage Ⅳ in NED status) setting, albeit with no evidence to support this approach. OS was not improved in the EORTC 40983 but the enrollment of patients was less than originally expected and its statistical power was called into question. Two other studies were reported in the adjuvant setting after complete resection of liver metastases[50]. They were also underpowered and employed outdated chemotherapy (5-FU bolus). The poor accrual in these clinical trials is most likely related to the oncologists' reluctance to enroll patients in studies that involved a surgery only arm. One single institution, single arm study showed 73% ORR (9% complete pathological response) in 56 patients treated with XELOX + bevacizumab in a peri-operative setting (6 doses pre- and 6 other post-surgery)[51]. The use of biological agents in the post-surgical period, when the patient is NED, is very controversial. Based on the results from adjuvant studies this practice should be discouraged. However, formal studies addressing this issue are missing. Other relevant issue with upfront resectable disease is the fact that chemotherapy could result in liver damage (*e.g.*, steatohepatitis) which could jeopardize patient's only curative chance.

A different scenario presents when the patient has liver-limited but unresectable metastases. Some of these patients (*e.g.*, low volume but abutting critical structures) have borderline disease, potentially amenable to be converted. In these cases, clinician should choose the best possible regimen to obtain maximal response rate. Before

the advent of the anti-EGFR and bevacizumab, conventional chemotherapy agents had already proven to enable surgical resection in a proportion of patients. Regimens such as FOLFOX or FOLFIRI have a conversion rate close to 40% and this could be improved with FOLFOXIRI[20,52,53]. The obvious question then is how much bevacizumab or the anti-EGFR drugs add to this and which one to use. A practical consideration is the fact that bevacizumab, which is the only option in KRAS mutant cases, has to be stopped at least 6-wk before surgery. For wild-type tumors, evidence may be slightly stronger for anti-EGFR drugs.

In the Germanic CELIM phase 2 study, 114 patients were randomly assigned to FOLFOX-6 or FOLFIRI, both regimens with cetuximab[54]. Patients required having technically unresectable liver metastases or more than five lesions. From a 106 evaluable patients, 36 of them (34%) had R0 resection but this proportion reached 60% in the wild-type KRAS population (41/68). Similar results were obtained in retrospective series. Even stronger evidence supporting the use of anti-EGFR in this particular setting came from a recently published Chinese study[55]. This phase 2, randomized study compared the efficacy of conventional chemotherapy (FOLFOX-6 or FOLFIRI) with or without cetuximab. Conversion to resection was the main outcome and after randomizing 138 patients the arm with cetuximab duplicated the proportion of patients deemed eligible for resection (13% *vs* 29%) and triplicated the R0 rates (7.4% *vs* 25.7%). Based on these reports chemotherapy plus cetuximab should be strongly considered for patients with wild-type KRAS and liver only metastases. Detractors of this posture may argue, though, that in a fresh head-to-head comparison between cetuximab and bevacizumab, ORR was not different (FIRE-3; see below).

Data supporting the use of bevacizumab in this scenario is somehow controversial. The most vigorous argument against its use comes from the previously mentioned NO16966 study[14]. There was no difference in ORR and there was similar proportion of patients attempted to have curative metastatectomies (8.4% *vs* 6.0%). However, the study was not designed to test this hypothesis. On the other hand, small phase 2 and retrospective studies brought up to 40% conversion rates and pathological responses when bevacizumab is added to XELOX, representing the fundaments for its use especially in KRAS mutant patients[56,57]. In that regards, the possibility of adding a stronger chemotherapy, such as FOLFOXIRI, should be seriously considered for fit patients.

Which is the ideal chemotherapy mate of the current monoclonal antibodies? And in patients with wild-type KRAS which strategy we should choose? Anti-VEGFR or Anti-EGFR?

Doublet chemotherapy is often used as upfront systemic treatment for advanced CC. It is unclear to these days which doublet is better for each patient and this has to be individualized according to toxicity and comorbidities. FOLFOX, XELOX, and FOLFIRI appear to be similar in efficacy but with different toxicity profile. XELIRI is harder to endure. Most patients tolerate a chemotherapy doublet, but probably not all of them need it as showed by the frequently forgotten Dutch study (CAIRO-1)[58]. The addition of biologics has improved outcomes, but not as much as we hoped. When KRAS is mutated, the chemotherapy chosen must be accompanied with bevacizumab. The dilemma starts with the K-RAS wild type patients. There are clinical trials showing benefit for both approaches: anti-VEGFR and anti-EGFR. The question is which patient would benefit from one or the other schema.

As previously mentioned, in the NO16966 study bevacizumab extended PFS by 1.4 mo, with a more profound effect seen in the XELOX arm[13]. But, why bevacizumab had such a discrete effect on PFS? Was this due to no synergistic or additive effect with FOLFOX/XELOX? The answer is NO, since FOLXOX + bevacizumab is active, even in second line with significant prolongation of OS[12]. Some authors advocate the idea of failure due to the "OPTIMOX" effect, meaning when neurotoxicity occurred oxaliplatin was stopped and fluoropyrimidine plus bevacizumab was continued until progression. This could be the case, since when we observe the difference in PFS of the patients on treatment, this is much more important. It is also feasible that bevacizumab works better with "inferior chemotherapies" such as IFL and have less to offer with "superior chemotherapies" such as XELOX or FOLFOX.

Regarding the anti-EGFR therapies, the earlier cited CRYSTAL and PRIME studies are the foundations for its use in the frontline treatment[40,41]. Nonetheless, in 2011 the COIN study was published[59]. With 2445 KRAS wild-type patients randomized to XELOX or FOLFOX +/- cetuximab, the COIN study represents the biggest trial ever conducted in this population. The results were disappointing. No difference in PFS was seen. Shortly thereafter, the results of the NORDIC VII were released[60]. Patients were randomly assigned to either standard Nordic FLOX or cetuximab + FLOX or cetuximab + intermittent FLOX. The median PFS was 7.9, 8.3, and 7.3 mo respectively and was not significantly different. In patients with KRAS wild-type tumors, cetuximab did not provide any additional benefit but in patients with KRAS mutations a trend toward worsening PFS was observed. The authors concluded that cetuximab did not add significant benefit to the Nordic FLOX regimen as first-line treatment. Additionally, the randomized, phase 2, PEAK study was presented in the 2013 ASCO GI Meeting[61]. This study enrolled 285 patients and evaluated the use of first-line mFOLFOX-6 + panitumumab *vs* bevacizumab. Again, no difference was observed. It is confusing how to interpret the actual role of anti-EGFR and chemotherapy since COIN, the largest phase 3 randomized trial, was negative. The NORDIC was a negative trial as well, but in the scenario of 5-FU given by bolus, a seldom used strategy nowadays.

It is possible that irinotecan-based chemotherapy would be necessary when anti-EGFR is considered in the treatment of metastatic disease. It is also curious that the hazard ratios for PFS with anti-EGFR antibodies tend to become more significant as the number of previously used lines of treatment upsurges. For instance, these agents are useless in the adjuvant setting and grow more active as disease progresses (*e.g.*, 3rd line).

Lastly, the FIRE-3 trial was presented in June 2013[62]. This was a randomized multicenter trial comparing the efficacy of FOLFIRI + cetuximab *vs* FOLFIRI + bevacizumab in patients with wild-type KRAS metastatic colon cancer. The primary endpoint was ORR and 592 patients were included. The study was negative for its primary end-point, with comparable ORR (62% *vs* 58%, $P = 0.183$). Significantly better PFS and OS were seen in the FOLFIRI + cetuximab arm (28.8 mo *vs* 25.0 mo; $P = 0.016$) although this was a secondary endpoint. A preplanned analysis of the FIRE-3 was presented at the European Cancer Congress 2013, aimed to investigate the effect of several other mutations beyond the exon 2 as well as BRAF (V600E)[63]. About 15% of patients were found to have these extra mutations. This sub-analysis incorporated 342 KRAS wild-type patients and 178 KRAS mutant patients (113 with exon 2 mutations plus the 65 newly identified patients). The subgroups were compared for ORR, PFS, and OS. Wild-type patients had 33.1 mo OS with FOLFIRI + cetuximab in comparison to 25.6 mo with FOLFIRI + bevacizumab (HR = 0.70; $P = 0.011$). In KRAS-mutant patients, this difference was not observed. No difference in PFS was seen in the KRAS wild-type group ($P = 0.54$), but interestingly for KRAS-mutated patients PFS was better in the bevacizumab arm (12.2 mo *vs* 6.1 mo; $P = 0.004$). ORR was similar between the arms, irrespective of KRAS status. It is difficult to understand why a treatment that does not improve ORR and PFS could show such an impact on OS.

In conclusion, in 2014 we have only one approach for KRAS mutated tumors which is chemotherapy plus bevacizumab. For KRAS wild type we can use either chemotherapy plus anti-EGFR antibodies OR chemotherapy plus bevacizumab. Going deeply into this last category, at least one clinical trial suggested cetuximab + FOLFIRI as the possible best option. However, head-to-head comparison with FOLFOX+B is lacking and this still represents a valid option. We disfavor oxaliplatin-based chemotherapy with cetuximab based on the MRC COIN study.

Which is the best strategy after progression with bevacizumab-containing regimen? Switch chemotherapy and keep anti-VEGFR or switch to anti-EGFR antibodies?

Preclinical data showed that continuous VEGF inhibition prevents tumor regression[64]. However, risk-benefit ratio associated with continuing bevacizumab use after initial progressive disease was unknown. In 2008, Grothey *et al*[65] reported a novel observation gathered from the BRiTE study. In this large, observational cohort study patients were classified according to the treatment received once they progressed to first line bevacizumab containing regimens. Three groups were identified; those with no post-progression treatment, those who received no-bevacizumab related treatment and those who continued bevacizumab beyond progression. When adjusted for other variables, bevacizumab beyond progression was associated with longer survival ($P < 0.001$). Based on the hypothesis generated by the BRiTE investigators, a randomized phase Ⅲ study-ML18147 trial-was launched[66]. The investigators assessed continuation bevacizumab plus second-line chemotherapy (no anti-EGFR) after standard first-line bevacizumab-based treatment. Bevacizumab lead to a 1.4 mo longer OS (11.2 mo *vs* 9.8 mo; $P = 0.006$).

At the present time is unclear how to proceed in patients who are treated with bevacizumab-containing chemotherapy who progress. In the KRAS/NRAS mutated patients the concept is to maintain the anti-angiogenic status in a similar strategy as the one employed in HER-2/Neu positive breast cancers[67]. This could be achieved either by keeping bevacizumab and changing the chemotherapy regimen or by switching to ziv-aflibercept and irinotecan containing regimen. For wild type tumors, the same options applied but anti-EGFR monoclonal antibodies should be strongly considered because it is important to emphasize that independently of the biological agent chosen first, once progressed patients with wild type tumor should be able to receive all agents sequentially[68].

NEW TARGETS

In the previous sections we have focused on the evidence behind what is currently considered the state of the art treatment of metastatic colon cancer. However, since this field is quite dynamic and the frontiers are in continuous expansion, it will be appropriate to discuss some of the new strategies that are currently being investigated. For description purposes, we will subdivide them based on its main mechanism of action.

Intracellular anti-EGFR therapies

Monoclonal antibodies block the extracellular domain of EGFR. Tyrosine kinase inhibitors (*e.g.*, erlotinib or gefitinib) target the intracellular domain of the receptor. Unlike lung cancer, EGFR mutations are rarely found in colon cancer and are usually not associated with response[69]. Moreover, positive EGFR protein expression does not predict response to treatment[70]. Results have been generally disappointing with no objective responses seen with erlotinib and no improvement in OS with the combination of gefitinib and FOLFIRI[71,72]. However, and after many previous unsatisfactory attempts, a positive study was finally published. Tournigand and colleagues recently presented the results of the phase 3 DREAM trial (OPTIMOX Ⅲ) showing that the addition of erlotinib to bevacizumab maintenance therapy after induction with chemotherapy + bevacizumab resulted in a small, but statistically

significant improvement in PFS from 4.6 to 5.8 mo (P = 0.005)[73]. Remarkably, KRAS mutation status was not a determinant of efficacy and patients with KRAS mutated had even better results. Some clinical trials are currently assessing the role of dual EGFR blocking (panitumumab + erlotinib) with or without chemotherapy in patients with progressed KRAS wild type tumors (NCT00940316). This approach is attractive especially in patients with poor performance status. Nonetheless, it will be at least 1 or 2 years before results become available.

BRAF inhibitors

Vemurafenib targets the BRAF V600E mutation and was proved to be effective in advanced melanomas. Unfortunately, results have been elusive in stage Ⅳ colon cancer. In a small phase I study in patients with BRAF mutant metastatic disease, only 1 of 19 patients had a partial response with single agent vemurafenib[74]. Apparently, blocking the BRAF pathway causes a reflective hyperactivation of the EGFR pathway. For that reason, there seems to be some rationale in combining BRAF and EGFR inhibitors and in preclinical studies a synergistic effect was found[75]. An ongoing trial is evaluating the combination of vemurafenib and cetuximab (EUDRACT # 2011-004426-10).

Pi3K pathway

PTEN loss has been associated with worse survival outcomes in colon cancer[76]. Some studies have also shown that PIK3CA mutations and PTEN loss are associated with an absence of response to anti-EGFR therapies[77]. Aspirin seems to be able to block the PI3K pathway. In a recent retrospective study only patients with PIK3CA mutant but not wild-type colorectal cancers who took daily aspirin had better cancer-specific and OS than those who did not take aspirin[78]. A phase 2 trial combined capecitabine plus perifosine (an inhibitor of the PI3K/Akt/mTOR pathway) with promising activity; however the phase 3 was negative[79]. Additionally, the combination of MEK and PI3K/mTOR inhibitors is currently being evaluated in a phase 1 trial (NCT 01390818) and Hochster *et al*[80] recently reported stimulating results with the combination of selumetinib (MEK inhibitor) and irinotecan.

HER-2 pathway

Few studies, with inconsistent results, investigated the role of HER-2 gene amplification as a potential predictive factor for anti-HER2 therapy. Some reported that HER-2 amplification was associated with resistance to cetuximab and worse PFS or OS; others found neither predictive nor prognostic value in HER-2[81-82]. A phase 2 study evaluating the combination of FOLFOX and trastuzumab in patients who have progressed after 5-FU and/or irinotecan-containing therapy was recently concluded; results are pending (NCT00006015).

Antiangiogenics

In addition to bevacizumab and ziv-aflibercept, other anti-angiogenic drugs have been evaluated with mixed results. Cediranib, a VEGFR inhibitor, showed comparable efficacy to bevacizumab but was associated with increased toxicity[83]. A dual EGFR and VEGFR inhibitor, vandetanib, was ineffective[84]. Ramucirumab, an anti-VEGFR-2 monoclonal antibody, is currently under evaluation in a phase 3 (NCT01183780) following promising results in a phase 2 study[85]. Since there is no real validated marker to predict response to anti-angiogenic drugs, it may take some time before any other anti-angiogenic compound make it to the market.

Insulin growth factor axis

The insulin growth factor (IGF) cascade activates a number of intracellular signaling pathways, including the Ras/Raf/MAPK pathway and the PI3K/Akt pathway[86]. Consequently, it is a potential target for a number of drugs. The main drugs developed as IGF inhibitors have been monoclonal antibodies. Dalotuzumab failed at an interim analysis of a phase 2/3 trial but pre-specified biomarker analysis suggested that patients with higher levels of IGF-1 may be a small subgroup who would potentially benefit from this treatment. Consequently, this hypothesis is being evaluated in a phase 2 study (NCT01609231).

Immunotherapy

In spite of the tremendous excitement raised by innovative immune-therapies in other solid tumors the scenario in metastatic colon cancer has been quite frustrating. No responses were seen in early phase trials with ipilimumab[87]. The same occurred with anti-PD-1 antibodies[88]. Currently, some investigators are testing the use of vaccines (NCT01322815). However, colon cancer seems to remain indifferent against this immunological "rush" or "fever" that we are living at this moment.

CONCLUSION

In conclusion we can affirm that over the last couple of years we have made some small but consistent progress against colon cancer. Anti-angiogenic and anti-EGFR strategies have given dividends by prolonging PFS and to a lesser extend prolonging life in patients with metastatic disease. We are still learning how to use them and it may take time before we discover the best sequence and combination. We also expect that in the near future better biomarkers lead us to the deeply desire but still evasive personalized medicine. But beyond these small victories, new horizons are envisioned. For example, half of the patients have KRAS/NRAS mutant tumors, though there are few drugs that target RAS directly. However, bypassing agents such as MEK inhibitors either alone or in combination with PI3K inhibitors may show promising results. It is impossible to predict the future, but it is expectable and even desirable that soon this review will become obsolete. That is human nature. That is progress. And that is why we must force ourselves to keep us continuously updated.

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ORIGINAL ARTICLE

Novel diet-related mouse model of colon cancer parallels human colon cancer

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Abstract

AIM: To investigate the close parallels between our novel diet-related mouse model of colon cancer and human colon cancer.

METHODS: Twenty-two wild-type female mice (ages 6-8 wk) were fed the standard control diet (AIN-93G) and an additional 22 female mice (ages 6-8 wk) were fed the control diet supplemented with 0.2% deoxycholic acid [diet + deoxycholic acid (DOC)] for 10 mo. Tumors occurred in the colons of mice fed diet + DOC and showed progression to colon cancer [adenocarcinoma (AC)]. This progression is through the stages of tubular adenoma (TA), TA with high grade dysplasia or adenoma with sessile serrated morphology, intramucosal AC, AC stage T1, and AC stage T2. The mouse tumors were compared to human tumors at the same stages by histopathological analysis. Sections of the small and large intestines of mice and humans were evaluated for glandular architecture, cellular and nuclear morphology including cellular orientation, cellular and nuclear atypia, pleomorphism, mitotic activity, frequency of goblet cells, crypt architecture, ulceration, penetration of crypts through the muscularis mucosa and presence of malignant crypts in the muscularis propria. In addition, preserved colonic tissues from genetically similar male mice, obtained from a prior experiment, were analyzed by immunohistochemistry. The male mice had been fed the control diet or diet + DOC. Four molecular markers were evaluated: 8-OH-dG, DNA repair protein ERCC1, autophagy protein beclin-1 and the nuclear location of beta-catenin in the stem cell region of crypts. Also, male mice fed diet + DOC plus 0.007% chlorogenic acid (diet + DOC + CGA) were evaluated for ERCC1, beclin-1 and nuclear location of beta-catenin.

RESULTS: Humans with high levels of diet-related DOC in their colons are at a substantially increased risk of developing colon cancer. The mice fed diet + DOC had levels of DOC in their colons comparable to that of humans on a high fat diet. The 22 mice without added DOC in their diet had no colonic tumors while 20 of the 22 mice (91%) fed diet + DOC developed colonic tumors. Furthermore, the tumors in 10 of these mice (45% of mice) included an adenocarcinoma. All mice were free of cancers of the small intestine. Histopathologically, the colonic tumor types in the mice were virtually identical to those in humans. In humans, characteristic aberrant changes in molecular markers can

be detected both in field defects surrounding cancers (from which cancers arise) and within cancers. In the colonic tissues of mice fed diet + DOC similar changes in biomarkers appeared to occur. Thus, 8-OH-dG was increased, DNA repair protein ERCC1 was decreased, autophagy protein beclin-1 was increased and, in the stem cell region at the base of crypts there was substantial nuclear localization of beta-catenin as well as increased cytoplasmic beta-catenin. However, in mice fed diet + DOC + CGA (with reduced frequency of cancer) and evaluated for ERCC1, beclin-1, and beta-catenin in the stem cell region of crypts, mouse tissue showed amelioration of the aberrancies, suggesting that chlorogenic acid is protective at the molecular level against colon cancer. This is the first diet-related model of colon cancer that closely parallels human progression to colon cancer, both at the histomorphological level as well as in its molecular profile.

CONCLUSION: The diet-related mouse model of colon cancer parallels progression to colon cancer in humans, and should be uniquely useful in model studies of prevention and therapeutics.

Key words: Diet; Deoxycholate; Mouse model; Colon cancer; Histology; Chlorogenic acid; 8-OH-dG; Beclin 1; Beta-catenin

Core tip: Mouse models of colon carcinogenesis are essential as platforms for trials of prevention and therapy. However, most previous rodent models of colon carcinogenesis lack an invasive phenotype and/or do not share several significant genetic events and histopathological features of human colon cancer. This new diet-related mouse model of colon cancer is unique in being closely parallel to human progression to sporadic colon cancer by measures of its histomorphology and its molecular profile. It also has a natural basis, using dietary deoxycholic acid, long thought to be a central causative agent in colon carcinogenesis.

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INTRODUCTION

Epidemiological studies show that rates of colon cancer incidence and mortality vary substantially across regions of the world. The rate of colon cancer incidence differs between countries by more than 10-fold[1]. More dramatically, Native Africans in South Africa have a colon cancer rate of < 1:100000[2] compared to the incidence rate for male African Americans of 72:100000[3]. In populations migrating from low-incidence to high-incidence countries rates change rapidly, and within one generation may reach the rate in the high-incidence country. This has been observed, for instance, in the colon cancer incidence of migrants from Japan to Hawaii[4]. These changes in colon cancer rates are thought to be largely due to changes in diet. Large increases in both meat and fat in the diet correlate with large increases in rate of colon cancer, graphed on an exponential scale[5].

In populations with a high incidence of colorectal cancer, fecal concentrations of bile acids are increased[6,7], suggesting that increased exposure of the colonic lumen to high levels of bile acids plays a role in the natural course of development of colon cancer. For example, the concentration of deoxycholic acid (DOC) in the feces of Native Africans in South Africa is 7.30 nmol/g wet weight stool while that of African Americans is 37.51 nmol/g wet weight stool, so that there is 5.14 fold higher concentration of DOC in stools of African Americans than in Native Africans[8]. As indicated above, there is a more than 72-fold greater rate of colon cancer in African American males than in Native Africans of South Africa. The hydrophobic bile acids, DOC and lithocholic acid, appear to be the most significant bile acids with respect to human colorectal cancer[6].

Since the bile acid DOC was implicated as important in colon cancer etiology in humans, we previously investigated whether DOC, at a high human physiologic level, could be a colon carcinogen in an experimental mouse model[9], and found that a high human physiologic level of DOC in the mouse colon does indeed cause colon cancer. We investigate, in the current study, whether the progression to colon cancer due to high physiologic levels of DOC in the mouse, by the gold standard histomorphologic analysis[10], is closely parallel to progression to colon cancer in humans. Other studies indicate that preneoplastic areas (field defects) are altered in molecular markers in human progression to colon cancer. We evaluate four of these markers: 8-OH-dG, ERCC1, beclin-1 and beta-catenin in the mouse colon progressing to colon cancer.

MATERIALS AND METHODS

Animals

Wild-type female B6.129PF2/J mice, ages 6-8 wk old, were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were the second generation (F2) of a cross between two well-established, inbred, wild-type strains: C57BL/6J and 129S1/SvlmJ (one of which carried a recessive albino mutation). The phenotypes of these F2 wild-type mice is expected to be varied, since the contribution of the two parental wild-type strains will be different in each F2 offspring, as illustrated by the color variation in these mice (Figure 1). It was intended that these mice be similar to a normal healthy human population in their genetic variation. Mice were main-

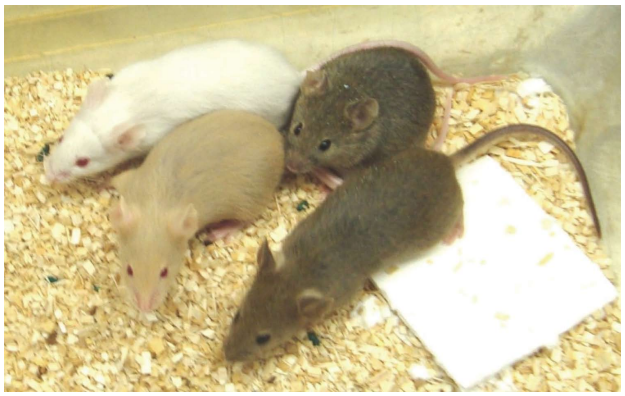

Figure 1 Young mice from 2nd generation cross of 2 wild type inbred lines show variation in colors.

tained at the University of Arizona's Animal Care Facility. All animals were raised, starting with 4 mice in each pan, in cages under nonsterile microisolator conditions and in compliance with the regulations and NIH guidelines for Care and Use of Laboratory Animals. All mice were weighed and their weights recorded weekly.

The mice were free of murine viruses, pathogenic bacteria (including *Helicobacter spp.*), and endo- and ecto-parasites by routine health evaluations. The mice were maintained on a 12-h light-dark cycle with water ad libitum and fed the control AIN-93G diet (Table 1), either unsupplemented or supplemented with 0.2% DOC. Purified diets were prepared as needed by Harlan Teklad, Madison, WI (including the DOC-containing diet). DOC was supplied by Sigma-Aldrich Corp, St. Louis, MO. Mice were first fed the control diet for 2 wk for acclimation. Then half the mice were fed with diet + DOC and half with control diet alone. Ten months after being switched to their experimental diets the mice were sacrificed, using CO_2. At the time of being placed on the experimental diets, 24 mice fed the control diet and 24 mice fed diet + DOC each consisted of 6 mice 6 wk old, 15 mice 7 wk old, and 1 mouse 8 wk old. During the succeeding 10 mo, 2 mice from each group died of unknown causes so that 22 mice in each group completed the experiment.

Histopathology, gross and microscopic images of human tissue

Before any biopsy tissue samples were obtained during colonoscopy, informed consent was given by the patient, using a form approved by the University of Arizona Institutional Review Board. Biopsy specimens were completely fixed in 10% buffered formalin for 6 to 12 h, followed by routine processing through graded alcohols and subsequent embedding into paraffin blocks. Tissue samples from colonic resections were obtained after informed consent before surgery. Colonic segments were cut open and gross photographic images of colonic tumors and polyps were obtained. Adequate representative tissue samples were obtained from areas of tumors and adjacent colonic mucosa. Similar to the biopsy specimens, these tissue samples were fixed in 10% buffered formalin for 24 to 36 h, transferred to graded alcohols, followed by paraffin embedment.

Table 1 AIN-93G diet composition

Ingredients	Percentage
Corn starch	39.75%
Casein vitamin free	20%
Maltodextrin	13.20%
Sucrose	10%
Soybean oil	7%
Powdered cellulose	5%
AIN 93G mineral mix	3.50%
AIN 93 vitamin mix	1%
L-cystine	0.30%
Choline bitartrate	0.25%
t-butylhydroquinone	0.0014%

Three 4-micron tissue sections were cut from all retained paraffin-embedded tissues. The tissues were then placed on glass slides, stained with hematoxylin and eosin, and subjected to histopathologic analyses. Morphologic evaluation was performed using a brightfield digital light microscope (Motic BA300).

Histopathology, gross and microscopic images of mouse tissue

The gastrointestinal (GI) tracts of mice, including rectum, colon, cecum, small intestine, stomach and lower esophagus, were removed, opened longitudinally, rinsed with phosphate-buffered saline (PBS) and divided into sections that could fit into paraffin blocks. All parts of the lower GI tract including rectum, colon and cecum were retained for fixation and paraffin embedment and any parts of the small intestine, stomach and esophagus that had a visible protrusion were retained. In addition, other organs including liver, pancreas, spleen, breasts and lymph nodes near breasts were examined, and if there were any potentially aberrant areas observed, sections of these organs were also retained. All retained sections were placed flat on Matricel membranes for good orientation. Segments of intestine with grossly visible mucosal nodules were photographed with a Sony Cybershot 7.2 megapixel camera. Sections were subsequently fixed in 10% formalin overnight at 4 ℃, then transferred to 70% alcohol, and embedded in paraffin.

Three to six 4-micron tissue sections were cut (multiple sections were cut to ensure any tumors or aberrant areas were included in the sections) from all retained tissues. The tissues were then placed on slides, stained with hematoxylin and eosin, and assessed for histopathologic characteristics. Morphologic evaluation was made on all the tissues on slides, using a brightfield digital microscope (Motic BA300). There is currently no accurate substitute for histopathologic determination of colonic neoplasia[10].

Diagnosis of histopathology

Anil R Prasad, MD, a surgical and cytopathologist with years of experience in GI pathology and immunohistochemistry diagnosed all of the tumors detected on the

basis of histopathologic criteria. The mouse tumors were compared to human tumors at the same stages by histopathological analysis. Sections of the small and large intestines of mice and humans were evaluated for glandular architecture, cellular and nuclear morphology including cellular orientation, cellular and nuclear atypia, nuclear enlargement, hyperchromasia, chromatin clearing, pleomorphism, presence of nucleoli, atypical mitotic activity, frequency of goblet cells, crypt architecture, ulceration, invasion of malignant glands through the muscularis mucosa and submucosa and presence of infiltrating malignant glandular crypts within the muscularis propria. Digital photomicrographs of representative sections were obtained using Motic Images Plus 2.0 software.

Immunohistochemistry

Protein expression was assessed using standard immunohistochemical methods[11,12], with variations as needed, described here. Briefly, formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections and floated on water, the tissue sections were picked up onto slides, deparaffinized, and then rehydrated.

Antigen retrieval for 8-OH-dG was performed by immersing slides in 4 mol/L HCl for 20 min at room temperature, rinsing in distilled water four times, transferring slides to 0.1 mol/L Borax for 5 min at room temperature, rinsing four times in distilled water and placing slides, twice, in PBS, pH 7.4, for 5 min.

For ERCC1, antigen retrieval was performed in citrate buffer (2.1 g citric acid + approximately 5 mL 5 mol/L NaOH + 1 L water, pH 6.1) brought to a boil in a microwave and then kept at high temperature for 6 min in the microwave followed by cooling on ice for 20 min. The slides were then washed with PBS for three minutes followed by a distilled water wash for three minutes.

Antigen retrieval for beclin-1 was performed by heating in a microwave in 0.1 mol/L citrate buffer (pH 6.1) and then cooling to room temperature.

For beta-catenin, antigen retrieval was performed in citrate buffer at pH 6.0, the slides were brought to a boil in a microwave and then kept at high temperature (not boiling) in the microwave for 10 min, followed by cooling on ice for 20 min. The slides were then washed with PBS for three minutes followed by a water wash for three minutes.

The slides were then rinsed with distilled water. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 30 min, and then the tissue sections were rinsed with distilled water and PBS. Next, slides were placed in Sequenza staining racks (Shandon Sequenza Immunostaining System from Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA) and rinsed with PBS.

For 8-OH-dG, a non-specific protein binding blocking step was used. For this, 150 μL 5% normal horse serum in PBS was added to each slide, which was allowed to stand at room temperature for 60 min. Next, without rinsing, 150 μL antibody against 8-OH-dG (QED 12501 from *QED Bioscience Inc.*, San Diego, CA) diluted with 2% BSA in PBS to 2 μg/mL was added to each slide and the slides were kept in the refrigerator at 4 ℃ overnight, followed by rinsing three times with PBS. Then 100 μL biotinylated secondary rabbit anti-mouse antibody (DAKO 0413) was added at a 1:400 dilution in 2% BSA in PBS, followed by incubation for 30 min at room temperature. At this point, Vectastain ABC reagent was prepared according to the manufacturer's instructions, and allowed to stand for 30 min before use. Then slides were rinsed with PBS three times, three drops of Vectastain ABC reagent were added and slides were incubated at room temperature for 30 min, followed by three rinses with PBS.

For ERCC1, 3 drops per slide of "Background Sniper" (from Biocare Mach 3 kit, Biocare Medical, Concord, CA) were added and left for 10 min at room temperature to reduce non-specific staining of background proteins. The ERCC1 slides were rinsed with PBS. Then a primary mouse monoclonal antibody was used (8F1 from Neomarkers, Freemont, CA). The mouse monoclonal antibody was added at 2 μg/mL in 2% BSA/PBS and left to incubate at room temperature for 45 min before three PBS washes. For the secondary antibody, the polyclonal rabbit anti-mouse Dako Biotinylated secondary antibody (E0413, DAKO Corp., Carpinteria, CA) was added at 120 μL/slide at a 1:300 dilution (in 2% BSA/PBS) and incubated for 30 min at room temperature before being rinsed 3 times with PBS. Vectastain Elite avidin-biotin complex method kit PK 6100 (Vector Laboratories, Inc., Burlingame, CA) was then used according to the manufacturer's instructions at 3 drops per slide and incubated at room temperature for 30 min before 2 rinses with PBS.

For beclin-1, to prevent nonspecific binding, the slides were blocked with 1.5% goat serum (Vector Laboratories, Burlingame) and then immunostained using a polyclonal anti beclin-1 antibody from ProSci Inc. (Poway, Calif, United States) at a concentration of 1 μg/mL. Sections were then incubated using a biotinylated antirabbit secondary antibody (Vector Laboratories) and Vectastain Elite ABC (Avidin Biotin Complex) reagent (Vector Laboratories).

For beta-catenin, first blocking serum consisting of 1.5% normal rabbit serum was prepared by adding 30 μL of normal rabbit serum to 2 mL BSA/PBS (prepared as 500 μL 22% BSA in 5 mL PBS) and then 120 μL was added per slide for one hour. Diluted beta catenin antibody (beta-catenin 610153, BD Biosciences San Jose, CA) was prepared by using beta catenin antibody at 250 μg/mL and diluting 6 μL into 1194 μL of 2% BSA in PBS. Without rinsing the slides, this antibody was added at 120 μL per slide for one hour. At this point, Vectastain ABC reagent was prepared according to manufacturer's instructions, and allowed to stand for 30 min before use. Then the secondary antibody was added. This was a 1:400 dilution of DAKO 0413 rabbit anti-mouse biotinylated IgG (5 μL DAKO per 1995 μL 2% BSA in PBS) (DAKO Corp., Carpinteria, CA), 120 μL per slide for 30 min, followed by three rinses with PBS. Then three drops of Vector ABC reagent was added per slide for 30 min, followed by two washes with PBS.

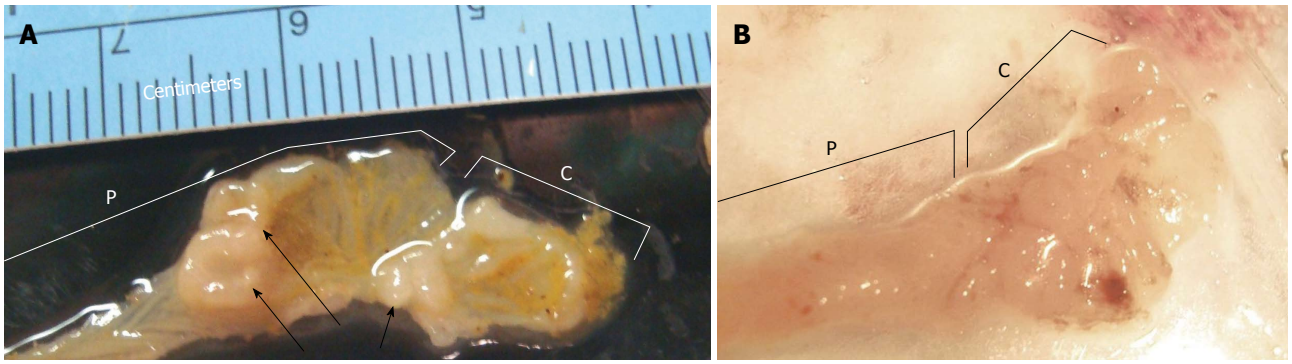

Figure 2 Opened proximal colons plus cecal areas of mice. A: 3 grossly visible mucosal nodules (arrows); B: No visible nodules. The letter P indicates a region of the proximal colon and letter C indicates a cecum.

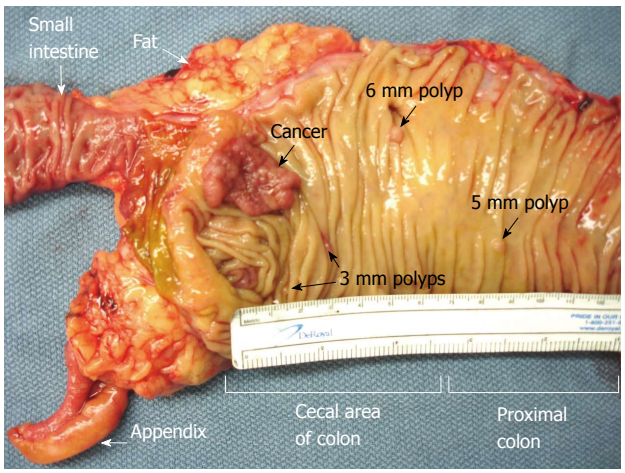

Figure 3 Cut open gross specimen of proximal human colon showing multiple tumors[13].

The slides were then removed from the Sequenzas, and color development was carried out by applying 0.025% diaminobenzidine tetrachloride (Sigma, St. Louis, MO) in PBS supplemented with 0.04% hydrogen peroxide. Sections were counterstained with 1:4 diluted hematoxylin (Sigma), dehydrated in a graded series of ethanols followed by xylene, and then mounted with coverslips using Cytoseal XYL (Richard Allen Scientific, Kalamazoo, MI). Brown staining indicates 8-OH-dG, ERCC1, beclin-1, or beta-catenin expression, and blue staining from hematoxylin identifies nucleoproteins in the nucleus.

Statistical analysis

Because the data was non-normally distributed, the non-parametric Mann-Whitney *U* test was performed to test for differences in occurrence of colonic and duodenal tumors and adenocarcinomas between mice fed diet + DOC and diet alone, and to determine if there were differences in the frequency of proximal and distal colonic tumors in the mice fed diet + DOC. To determine if there were correlations between mouse weight and number of tumors, a Pearson's correlation coefficient was calculated. The statistical analysis package Systat version 12 was used to analyze the data.

RESULTS

Gross physiology of mice fed diet + DOC

Mice fed the control diet and mice fed diet + DOC each looked healthy and were active during the entire time they were on their diets, even though the mice fed diet + DOC were almost all carrying neoplastic lesions (tumors, some of which were cancers) by 10 mo on the diet. This is similar to humans who have colon cancers, who also show no external signs until the cancers are very large or have metastasized.

Macroscopic phenotype of colorectum of mice fed diet + DOC or diet alone

Twenty out of the 22 female mice fed diet + DOC (91%) developed large macroscopically visible mucosal nodules (likely colonic neoplastic lesions). Figure 2 shows opened proximal regions of colons, including the cecums, of two mice fed diet + DOC. Figure 2A shows about 3 cm of proximal colon plus cecum in which three large mucosal nodules can be seen by eye. Histopathological examination of tissue from this area revealed three tubular adenomas, two of them with ulceration and one with high grade dysplasia. Figure 2B shows about 2 cm of another proximal colon plus cecum, and no mucosal nodules are seen. The colon of this mouse, also fed diet + DOC, had no colonic neoplasia at all upon histological examination.

None of the mice fed the control diet alone developed any colorectal tumors, evaluated both macroscopically and by microscopic histopathological examination of all rectum, colon and cecum segments.

Multiple tumors found in one location of the mouse colon, as in Figure 2A, indicate the presence of a field defect. By comparison, in humans, we also found multiple tumors in some of their much larger colon resections, and one example, showing 13 cm of the longitudinally-opened colon, is shown in Figure 3.

Macroscopic phenotype of small intestine of mice fed diet + DOC or diet alone

Most large mucosal nodules seen macroscopically in the

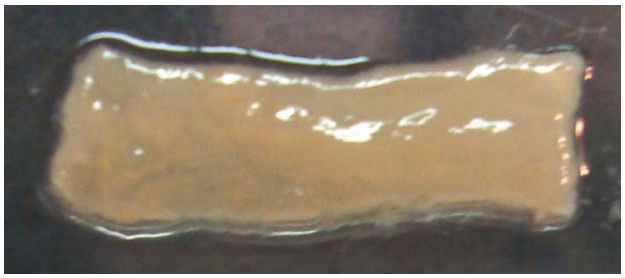

Figure 4 Opened segment of small intestine observed to have mucosal nodules.

large intestines of mice proved to be tumors upon histopathological examination. However, many small mucosal nodules were seen in the small intestine of each mouse, such as shown in Figure 4. Following microscopic examination, almost all were found to be benign Peyer's patches similar to those found in the human small intestine (Peyer's patches are gut-associated lymphoid tissue consisting of isolated or aggregated lymphoid follicles, and are the immune sensors of the intestine).

None of the small mucosal nodules in the small intestines of mice fed diet + DOC proved to be tumors. However, of the 22 mice fed control diet, 3 of the mice had small nodules that proved to be small adenomas. These small adenomas occurred near the Ampulla of Vater and Sphincter of Oddi (at the major duodenal papilla, in the second part of the duodenum), an area that experiences concentrated bile acids as they exit the common bile duct into the small intestine. This is the usual location of small intestinal tumors in humans, as well. These tumors were not cancers.

Types and locations of tumors

For each mouse fed diet + DOC, Table 2 lists data in 11 columns (Note that mice were 6 to 8 wk old when received, acclimated to the control diet for 2 wk, and then put on their diets for 10 mo, so that all mice, at termination, were 12 to $12^{1/2}$ mo old). In column 1, all 22 mice are listed by ascending weights. Columns 2 and 3 give the total number and location (distal or proximal) of all neoplastic lesions in these mice. There were 13 distal and 44 proximal lesions, for a total of 57 lesions.

Columns 4-11 give characteristics associated with the tumors enumerated in columns 2 and 3. Since any particular tumor may have two or more distinguishing characteristics, the total number of characteristics listed is greater than the total number of tumors. Column 4 indicates that two of the tumors in mouse 12 were hyperplastic. Hyperplastic polyps do not exhibit dysplasia and hence do not have malignant potential. Columns 5-8 give the characteristics of polyps exhibiting low and high grade dysplasia. There were 37 with tubular adenoma characteristics (TAs) (column 5), 15 with sessile serrated adenoma characteristics (SSA) (column 6), 17 of these adenomas (TA or SSA) had ulceration (column 7) and 3 adenomas displayed high grade dysplasia (HGD) (column 8). Columns 9-11 indicate characteristics of tumors that contain, or are entirely, clearly malignant and are at an early or later stages. These include 7 intramucosal adenocarcinomas (ACs) (an early stage) (column 9), 9 ACs at stage T1 (column 10) and 2 ACs at stage T2 (a late stage) (column 11). In total, 18 tumors were all, or in part, ACs. The polyps with low and high grade dysplasia (including those with ACs) totaled 55, or an average of 2.5 colonic neoplastic polyps or AC per mouse. The ACs often appreared to arise from a polyp with high grade dysplasia. For example, the mouse weighing 53.7 g had 7 tumors in the proximal colon, and one of these tumors was an SSA from which an AC had arisen and the area of the AC was ulcerated. Overall, 55 tumors were observed displaying morphological characteristics comprised of low and high grade dysplasia, or invasive malignancy of various stages.

Ten of the 22 mice had ACs, with some mice having more than one AC. There were 6 mice having just one AC, 2 mice having two ACs, 1 mouse having 3 ACs and 1 mouse having 4 ACs. Thus 45% of these 22 mice had at least one colonic AC after 10 mo of being fed diet + DOC.

Statistical analysis

As shown in Table 3, after 10 mo on the diet, 20 out of 22 (91%) of mice fed diet + DOC developed tumors (cancers or adenomas) in their colons, and of these diet + DOC fed mice, 10 (45%) had developed cancers. The 22 mice with no supplement to their diet had no cancers or adenomas in their colons. There was a significant difference in the number of mice with colonic tumor development between those mice fed diet + DOC and those fed diet alone (Mann-Whitney *U*, $P < 0.000001$ two-tailed). There was also a significant difference in the number of mice with cancer development between those mice fed diet + DOC and those fed diet alone (Mann-Whitney *U*, $P = 0.00042$ two-tailed).

Of the 57 total tumors found in the mice fed diet + DOC (Table 2), 44 (83%) were found in the proximal colon and 13 (23%) were found in the distal colon. There was a significant difference between the numbers of tumors in the proximal region and the distal region (Mann-Whitney *U*, $P = 0.0027$ two-tailed).

Three of the mice fed the diet only, with no supplement, had small adenomas near the Sphincter of Oddi (at the major duodenal papilla, in the second part of the duodenum). No mice in the DOC + diet group had adenomas in the duodenum. A Mann-Whitney *U* test to determine if there was a significant difference in occurrence of adenomas in the duodenum in the diet + DOC fed mice compared to the mice fed diet alone indicated that there was no significant difference ($P = 0.076$).

Histology of human and mouse colonic tissues compared

Pairs of adjacent images, Figures 5-8 below, illustrate the histomorphology of human and mouse colonic epithe-

Table 2 Mice fed diet + deoxycholic acid

Mouse weights (g)	Locations of tumors		Hyper-plastic polyp	Characteristics of polyps low and high grade dysplasia including those from which cancers arose				Stages of cancers found		
	Distal tumor	Proximal tumor		Tubular adenoma	Sessile serrated adenoma	Ulcerated adenoma	Adenoma with HGD	Intra-mucosal AC	Stage T1 AC	Stage T2 AC
18.7	3			3						
24		3		2			1	1		
25	2			2						
25.8	1				1	1			1	
25.9		3		3		2	1			
26.1		1		1		1				1
26.1	3			3		2			2	
27.3		2		2		1		1		
27.4		5		5		3		3		
28.8		2		2						
35.4	None	None								
35.7	2	3	2	3						
38.9	2	2		2	2					
40		3		2						1
41.1		4			3	3		1	2	
43		2			2	1				
43.1		1		1		1				
45.2		1		1		1	1			
45.2		2		2				1		
49.2	None	None								
53.7		7		3	4	1			4	
78.6		3			3					
Totals	13	44	2	37	15	17	3	7	9	2

AC: Adenocarcinoma; HGD: Highgrade dysplasia.

Table 3 Comparison of diet alone to diet + deoxycholic acid on colonic tumor and cancer development *n* (%)

Diet	Diet (mo)	Mice	Mice with tumors (adenomas + cancers)	Mice with cancer	Tumors (tumor burden[1])	Cancers (cancer burden[2])
Diet alone	10	22	0	0	0 (0)	0 (0)
Diet + DOC	10	22	20 (91%)	10 (45%)	57 (2.6)	18 (0.82)

[1]Tumor burden is the ratio of the number of tumors observed to the number of mice; [2]Cancer burden is the ratio of the number of cancers observed to the number of mice. DOC: Deoxycholic acid.

lial tissues. These Figures identify, in the legends and the images, the specific histomorphological characteristics that are crucial for characterizing either normal glandular architecture or identifiable stages in progression towards invasive adenocarcinoma. Stages shown include normal non-neoplastic glands (crypts) (Figure 5), tubular adenomas (Figure 6), tubular adenomas with high grade dysplasia (Figure 7) and sessile serrated adenomas (Figure 8). In each pair of tissues, the human and mouse crypts show closely parallel specifically identifying histomorphological characteristics. From the microscopic images alone, it is difficult to distinguish whether the tissues are from a human or from a mouse, though when viewed side-by-side, the mouse tissues are seen to have a smaller number of cells per crypt.

Figures 9 and 10 identify, in the legends and the images, the specific histomorphological characteristics that are crucial for characterizing invasive adenocarcinomas of stages T1 and T2. Figure 9A also shows some of the characteristics that may accompany colonic adenocarcinomas. In this image the adenocarcinoma arose in association with, or arose from a sub clone of, a sessile serrated adenoma. In addition, this adenocarcinoma shows ulceration of the colonic mucosa.

Only mouse tissues are shown in Figures 9 and 10, since human adenocarcinomas having penetration through the muscularis mucosa and entry into the submucosa could not be shown at the same magnification and still fit in the figure. These images were taken at intermediate magnification (10× objective lens), a lower magnification than the preceding images (taken with a 40 × objective lens).

Two examples of mouse colonic adenocarcinoma at low magnification (taken with a 4× objective lens) are shown in Figure 10. This magnification allows imaging of the majority of the cancers in single fields of view. Figure 10A shows a section through an entire cancer at stage T1 with mucosal ulceration, and Figure 10B shows a section through an almost entire cancer at stage T2.

Figure 11 shows portions of human and mouse stage

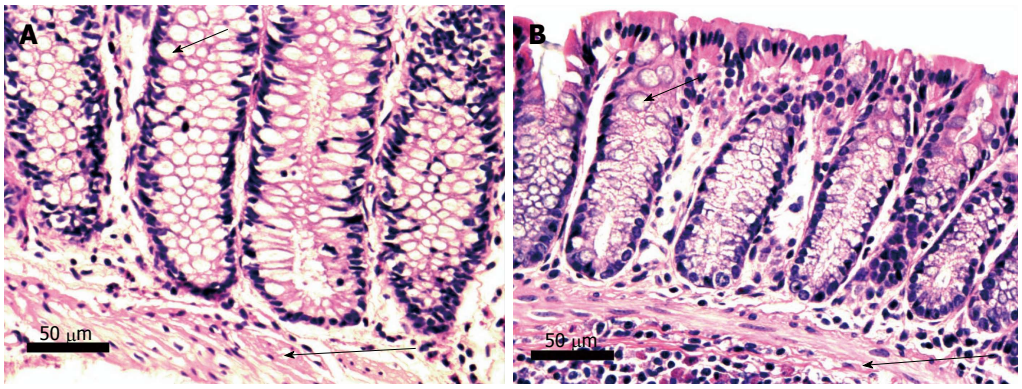

Figure 5 Histologically normal human (A) and mouse (B) colonic crypts, cut along the long axis of crypts. The normal human and mouse glands (crypts) are composed of columnar epithelial cells and goblet cells. Short arrows indicate typical goblet cells containing mucin (not stained, white in the image). About half of the cells in the crypts are goblet cells. Nuclei are darkly stained. All crypts are normally aligned colonic mucosal glands with the bases of the crypts abutting the muscularis mucosa. Long arrows indicate the muscularis mucosa. All crypt cells are parallel to each other and the nuclei are adjacent to each other, with no overlapping. Images obtained with 40× objective lens.

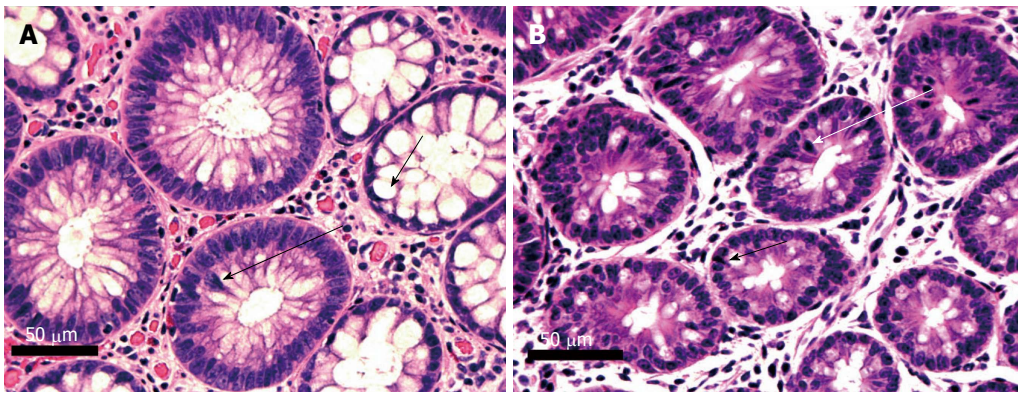

Figure 6 Human (A) and mouse (B) crypts cut across the short axis, showing tubular adenomatous crypts as well as histologically normal crypts. Crypts on the right in A and at the bottom of B have normal histology. Adenomatous crypts are seen to the left in A, and in the top half of B. Adenomatous glands show overlapping cells with hyperchromatic mitotically active nuclei (long arrows indicate examples of cells undergoing mitosis). Short arrows indicate typical goblet cells. The goblet cells in adenomatous glands are decreased in frequency compared to goblet cells in the histologically normal glands. Images obtained with 40× objective lens.

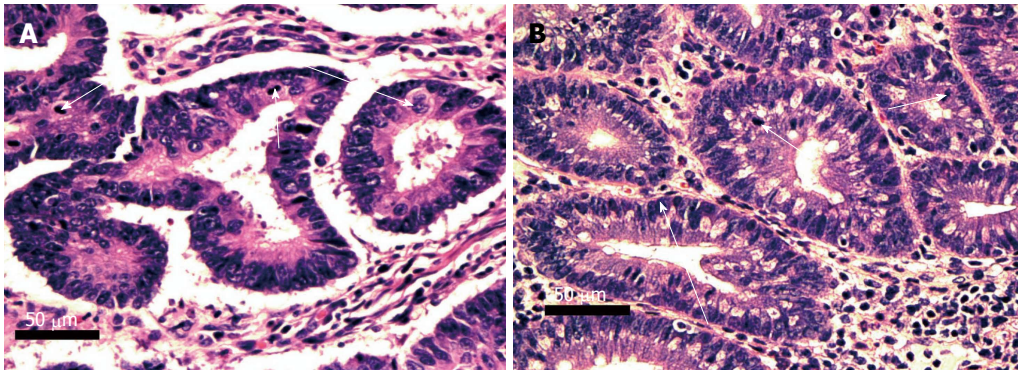

Figure 7 Crypts of tubular adenomas with high grade dysplasia cut across the short axis, human (A) and mouse (B). Glands with high grade dysplasia show overlapping cells with oval to round vesicular nuclei and prominent nucleoli (long arrows). Mitotic figures are abundant (short arrows). Complex architecture with infolding of crypts can also be seen. Images obtained with 40× objective lens.

T2 adenocarcinomas, showing adenomatous glands invading the muscularis propria. The presence of extravasated mucin, forming mucin pools adjacent to malignant glands are seen in Figure 11B.

IHC evaluation of molecular markers for progression to colon cancer

Tissues had been preserved in paraffin from our previous experiment where mice had been fed either the control diet,

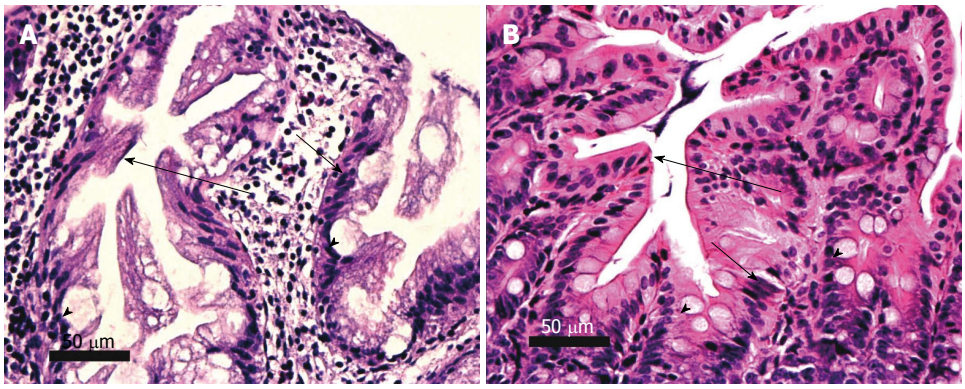

Figure 8 Sessile serrated adenomas, human (A) and mouse (B), cut along the long axis. Serrated glands show star shaped crypt architecture (long arrows). Adenomatous glands with hyperchromatic overlapping nuclei (short arrows) retaining goblet cells (arrow heads) are seen. Images obtained with 40× objective lens.

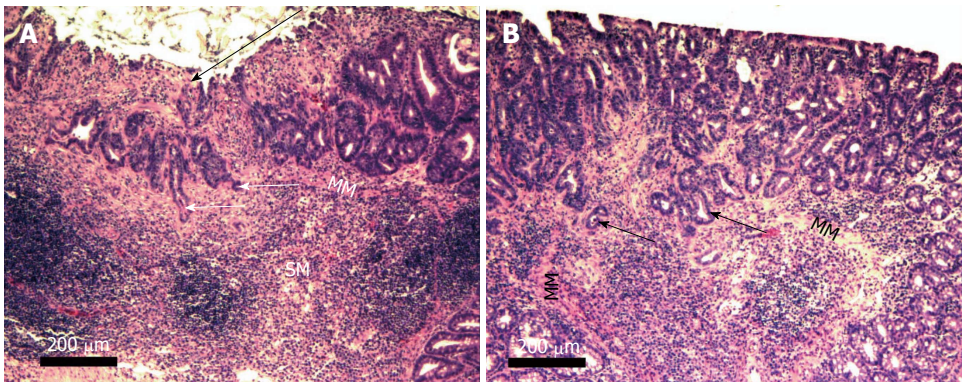

Figure 9 Two examples of mouse adenocarcinoma stage T1. A shows a sessile serrated adenoma in the right upper portion of the image and an ulcerated region (long arrow) above an adenocarcinoma that had penetrated the muscularis mucosa. Both A and B show invasive glands (short arrows) infiltrating through the muscularis mucosa (MM) into the submucosa (SM). Images obtained with 10× objective lens.

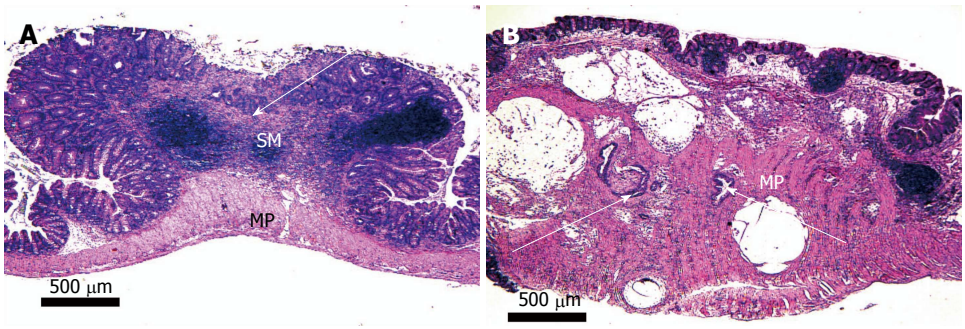

Figure 10 Mouse adenocarcinomas at stages T1 (A) and T2 (B). A shows a section through an entire cancer at stage T1, and B shows a section through an almost entire cancer at stage T2. A shows infiltrating malignant glands (long arrow) in submucosa (SM) but not in muscularis propria (MP). B shows infiltrating malignant glands (long arrows) within muscularis propria (MP). These adenocarcinomas are about 2 to 3 mm tall and about 6 mm wide and would correspond to the sizes of the mucosal nodules seen in Figure 2A. Pale areas in B are pools of mucin. Images obtained with 4× objective lens.

diet + DOC or diet + DOC + CGA[9]. From the colons of each of three mice on the different diets, a 4 micron tissue section was obtained and immunostained for location and level of a marker of progression to colon cancer. The segments of the colons evaluated were in regions of the colon without a neoplastic lesion. Thus, we were evaluating colon segments for the presence of preneoplastic areas from which a neoplastic lesion might be expected to arise. The small number of mouse colons evaluated constituted a brief survey of molecular markers altered in progression to colon cancer. The examples in Figures 12-16 were representative of the levels of biomarkers found, but with only three tissue samples, variation of the expression of each marker was not quantitated. As background information for these tissues, we note that in the previous experiment from which these tissues came, for the 12 mice fed the control diet none developed colonic neoplasia. For the 18 mice fed diet + DOC, 94% had developed colonic neoplasia,

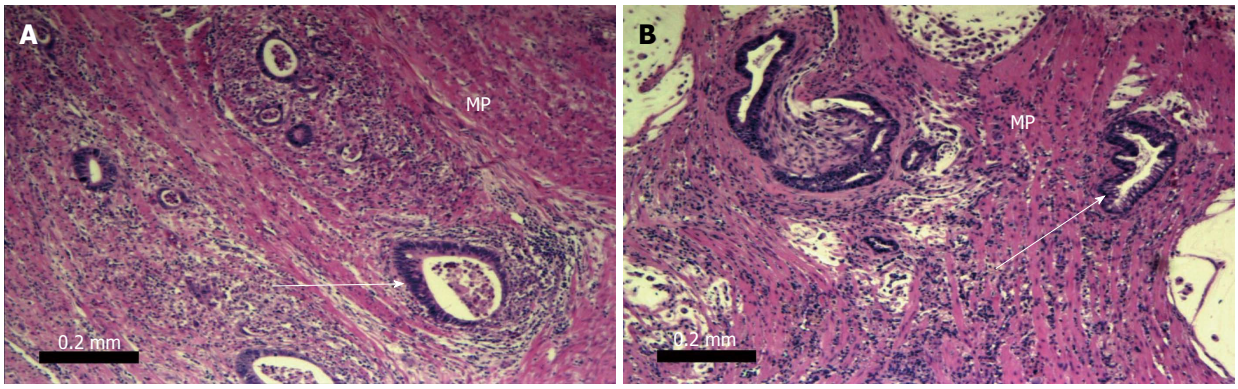

Figure 11 Invasion of the muscularis propria by adenocarcinoma stage T2, human (A) and mouse (B). Malignant glands (long arrows) can be seen invading the muscularis propria (MP). The pale areas within the stroma in B are mucin pools. Necrotic material is seen within the lumen of malignant glands in A and B. Images obtained with 10× objective lens.

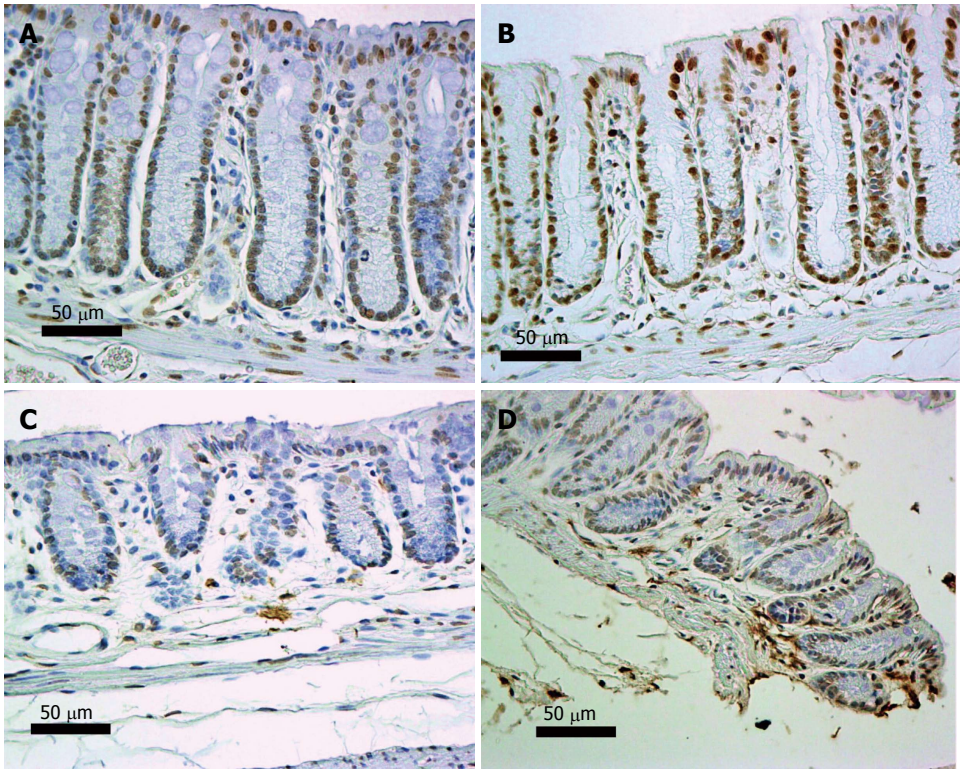

Figure 12 Colonic epithelia from mice fed diet + deoxycholic acid (A, B) or mice fed control diet (C, D) immunostained (brown) for 8-OH-dG, counter-stained with hematoxylin. Images obtained with 40× objective lens.

and for 56% of these mice the neoplasia had progressed to adenocarcinoma. There had been 12 mice fed diet + DOC + CGA, of which 64% developed colonic neoplasia, and for 18% of these mice the neoplasia had progressed to adenocarcinoma, so that CGA was somewhat protective against colonic neoplasia and adenocarcinoma

8-OH-dG in progression to colon cancer

As reviewed by Scott *et al*[14], the DNA damage 8-OH-dG is carcinogenic. Six mice, on their diets for 8 mo, were terminated and their colons removed for evaluation of nuclear 8-OH-dG (Figure 12). Three of these mice were on the standard diet and three had been fed diet + DOC. Colonic tissue sections from each mouse were placed on slides and immunostained for 8-OH-dG. Figure 12 shows tissues from 2 mice fed diet + DOC (Figure 12A and B) and 2 mice fed control diet (Figure 12C and D). Brown stain indicates 8-OH-dG and blue is hematoxylin stain for the chromatin in the nucleus. The level of 8-OH-dG was graded in the nuclei of the colonic crypt cells by IHC on a scale of 0-4. The nuclei of mice fed diet + DOC were largely at levels 3 to 4 (Figure 12A and B) while for mice fed diet alone were largely at levels 0 to 2 (Figure 12C and D). The images in Figure 12 were each uniform-

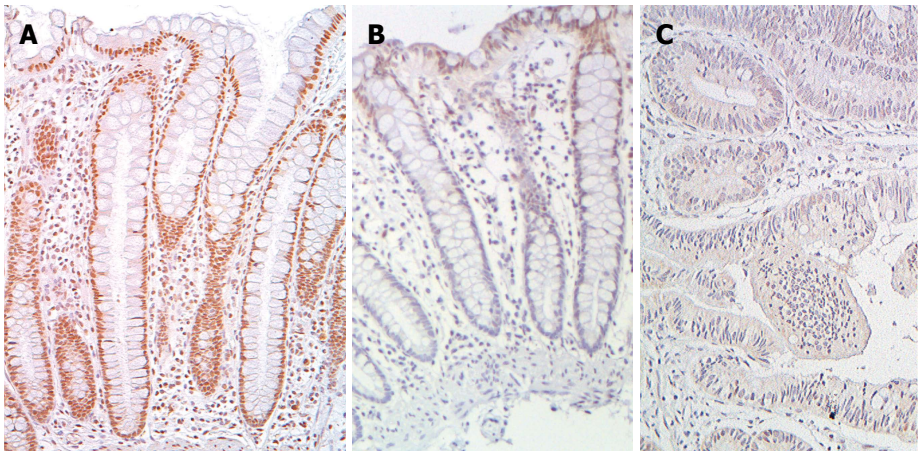

Figure 13 Human colonic mucosa immunostained (reddish brown) for excision repair cross-complementation group 1 with blue hematoxylin counter stain for chromatin. A: From patient without colonic neoplasia; B: From tissue near a colon cancer; C: From cancer tissue. Images with 40× objective. Scale shows 50 μm.

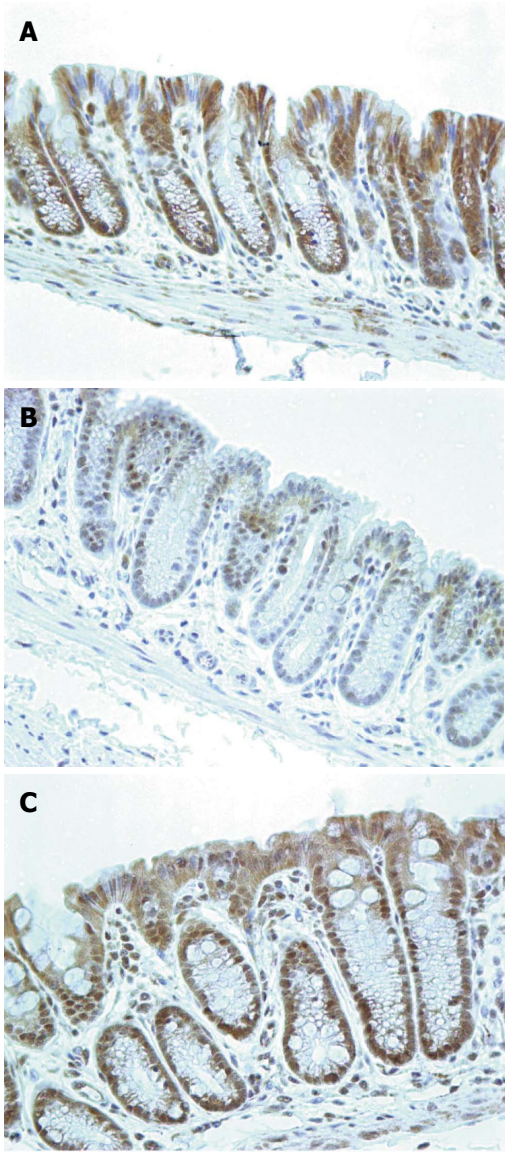

Figure 14 Mouse colonic epithelia with immunohistochemistry for excision repair cross-complementation group 1 (brown) and hematoxylin (blue) for chromatin. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid. Images obtained with 40× objective lens. Scale shows 50 μm.

ly enhanced in Paint Shop Pro 5 by increasing "shadow" to 35 and "saturation" to 35 to allow enhancement of the brown and blue colors for greater clarity in evaluating the immunohistochemical staining.

ERCC1 deficiency in progression to colon cancer

We recently reported that expression of DNA repair gene *ERCC1* was generally deficient in the histologically normal tissue surrounding human colon cancers (field defects susceptible to carcinogenesis) and in colon cancers themselves[11,12]. Figure 13 shows examples of IHC staining for ERCC1 of human colonic epithelia obtained during these previous studies. As shown in these images, the nuclei of cells in the colonic crypts of a patient without colonic neoplasm (Figure 13A) have high expression of ERCC1. However, in the crypts near a colon cancer (within 10 cm of a cancer in this example) (Figure 13B), cells in the lower parts of crypts (in the stem cell and proliferative regions) are usually deficient for ERCC1 while cells in the upper parts of the crypts and along the colonic lumen have restored ERCC1 expression. Within the area of a colon cancer (Figure 13C), ERCC1 is largely absent from the nuclei. The images in Figure 13 were each uniformly enhanced as described for Figure 12.

Nine mice, on their diets for 8 mo, were terminated and their colons removed for evaluation of expression of ERCC1. Three of these mice were on the standard diet, three had been fed diet + DOC and three had been fed diet + DOC + CGA. Colonic tissue sections from these mice were immunostained for ERCC1. Figure 14 shows typical colonic epithelial tissues from a mouse fed control diet (Figure 14A), a mouse fed diet + DOC (Figure 14B), and a mouse fed diet + DOC + CGA (Figure 14C). The colonic crypt cells of mice fed the control diet for 8 mo have high expression of ERCC1 (Figure 14A). For mice fed diet + DOC for 8 mo, cells in the lower parts of crypts are deficient for ERCC1 while the upper parts of the crypts usually have restored ERCC1 expression (Figure 14B). The cells of mouse colonic crypts of mice fed diet + DOC + CGA have high nuclear expression of ERCC1 (Figure 14C). The images in Figure 14 were each

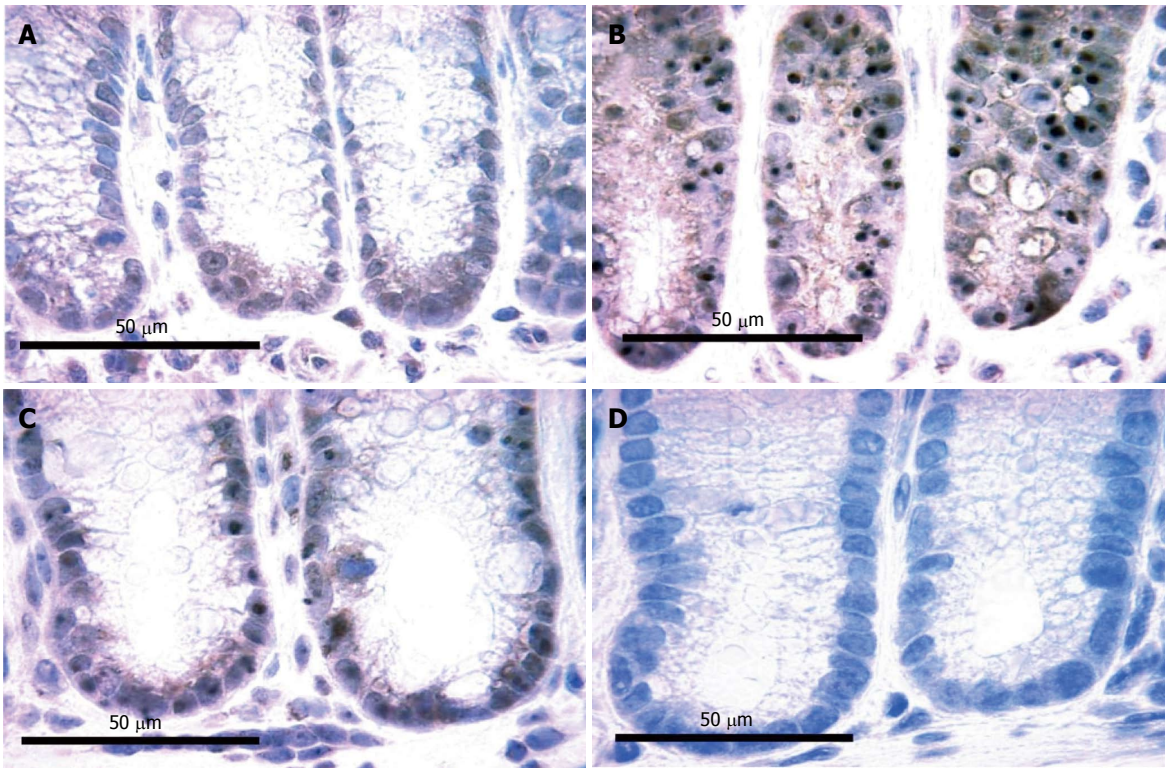

Figure 15 Immunohistochemistry of mouse colons for beclin-1. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid; D: Negative control without primary antibody (blue hematoxylin stain for nuclei). Images taken with 40× objective lens.

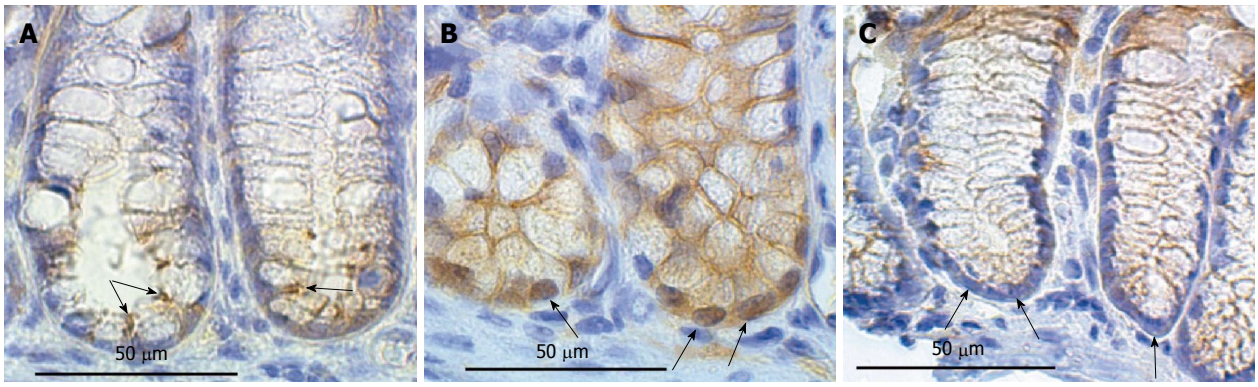

Figure 16 Lower regions of mouse colonic crypts immunostained for beta-catenin. Mice fed diets: A: Control; B: Diet + deoxycholic acid (DOC); C: Diet + DOC + chlorogenic acid (CGA). In A (control diet), in the stem cell region (lowest cells in the crypts), cells have beta-catenin expression localized to their membrane regions as shown by arrows. In B (diet + DOC), the stem cell region shows substantial nuclear localization of beta-catenin (arrows). In C (diet + DOC + CGA), stem cell region nuclei are largely deficient in beta-catenin, and the cytoplasm has low levels of beta-catenin, similar to the levels in mice fed the control diet alone. Images taken with 40× objective lens.

uniformly enhanced as described for Figure 12.

The mice fed the control diet had expression of ERCC1 (Figure 14A) that matched human ERCC1 expression for humans without colonic neoplasia (Figure 13A). The mice fed diet + DOC (and generally progressing to colonic neoplasia) had ERCC1 expression (Figure 14B) that matched human ERCC1 expression in a field defect giving rise to a cancer (Figure 13B). The mice fed diet + DOC + CGA, which had substantially fewer cancers[9], also had a level of ERCC1 expression (Figure 14C) that was similar to that of mice fed the control diet (Figure 14A).

Increased beclin-1 in progression to cancer

Beclin-1 is a central player in autophagy. The modulation of macroautophagy is now recognized as one of the hallmarks of human cancer cells[15]. Figure 15 shows colonic epithelium of mice immunostained for beclin-1, where the mice in Figure 15A-C were fed different diets for 8 mo. The level of beclin-1 was graded in the colonic crypt cells by IHC on a scale of 0-4. In the colonic epithelium of mice fed the control diet for 8 mo (Figure 15A) beclin-1 staining was at level 1. For mice fed diet + DOC (Figure 15B), expression was at level 4, and for mice fed diet + DOC + CGA expression was at level

Figure 17 Two mice, raised in the same pan, had different weights after 10 mo on their diet. The heavier mouse and the lighter mouse both appeared to be healthy and active.

3. For mouse colonic epithelium stained without the primary antibody (Figure 15D), staining was at level 0. These images were not enhanced.

Increased nuclear beta-catenin in the stem cell region in progression to cancer

The images in Figure 16 show the lower regions of mouse colonic crypts (including the stem cell regions) of mice that had been placed on three different diets for 8 mo - control diet, diet + DOC or diet + DOC + CGA. The colonic stem cell region showed only membrane expression of beta-catenin in samples of colonic epithelial tissue from all three of "control diet"-fed mice that were assessed here (one example is shown in the figure). The colonic stem cell region showed high nuclear expression of beta-catenin in samples of colonic epithelial tissue from all three of these diet + DOC fed mice that were assessed here (one example is shown in the figure). The colonic stem cell regions showed very low levels of beta-catenin in samples of colonic epithelial tissue from all three of the mice fed diet + DOC + CGA that were assessed here (one example is shown in the figure). The images in Figure 16 were each uniformly enhanced as described for Figure 12.

Weight distributions

The final weights of mice fed the control diet for 10 mo were quite varied, with the lowest weight being 25.2 g and the highest being 63.1 g. The mouse with the median weight was at 41.3 g. The distribution of weights for mice fed diet + DOC varied from 18.7 to 78.6 g, with a median weight of 35.5 g. Each mouse was weighed weekly, and no weight loss was detected for any of the mice during their 10 mo on each diet. Mice with relatively low weights at the end of 10 mo on their diets merely gained weight more slowly than heavier mice.

Each mouse, without respect to weight, appeared to be healthy and active (Figure 17). The variation in mouse weights, like the variation in colors of these mice (Figure 1), was likely due to the variation in their genetic constitutions. As pointed out in the Materials and Methods, the mice were the second generation (F2) of a cross between two well established, inbred, wild-type strains: C57BL/6J and 129S1/SvlmJ. The phenotypes of these F2 wild-type mice is expected to be varied, since the contribution of the two parental wild-type strains will be different in each F2 offspring. The varied weights of these mice may mimic the weight variations in the general human population.

A SKEW calculation on all the data had a value of 0.0896 indicating it was approximately symmetrically distributed. A *t* test was then applied to determine if there were significant differences between the weights of the control-fed and the diet + DOC-fed mice, using the assumption of unequal variances (since the variances were different). The two-tailed *t* test, which indicates if the differences between the two populations are larger or smaller than each other, gave a *P*-value of 0.159, indicating that there is no significant difference between the two populations in distributions of weight. An ANOVA analysis using the same datasets also gave a *P*-value of 0.159. Thus distributions of weights were similar and there was no significant difference between the weight distributions for the two types of diets. There was also no systematic association of type of tumor development with weight of the mice fed diet + DOC. A Pearson correlation analysis determined the weight of the mice fed a DOC-supplemented diet was not correlated to the number of colonic tumors found ($P = 0.78$).

DISCUSSION

Similarity of DOC in diet + DOC mouse colons to that of humans on a high fat diet

For humans on a non-controlled omnivorous diet in London England, the level of DOC in the feces averaged 3.2 mg/g dry weight[16]. A high fat human diet in the United States doubles the colonic DOC concentrations[17] and would subject people to colonic exposure to DOC at an average value in their feces of about 2 × 3.2 mg/g = 6.4 mg/g dry weight. Addition of 0.2% DOC for 6 mo to the diet of 18 wild-type male mice produced mouse feces with 4.6 mg DOC/g dry weight (comparable to the 6.4 mg/g dry weight for humans on a high fat diet). Mice on a control diet for 6 mo, on the other hand, had feces with less than a tenth the level of fecal DOC, having 0.3 mg DOC/g dry weight. Among the 18 mice fed diet + DOC, 17 developed colonic tumors in our previous study[9], including 10 mice with colon cancers. In our present study, using female mice instead of male mice, we confirmed a high frequency of colon cancer (10 of 22 mice) with mice fed diet + DOC.

Parallel histology of mouse model colon tumors and human colonic tumors

Histopathologic evaluation constitutes the gold standard for determining progression of colonic epithelium to colon cancer, to which other methods are compared[10]. Using histopathologic evaluation, we showed that mice fed diet + DOC progress to colon cancer in a manner closely

similar to such progression in humans.

We found that tumors in these diet + DOC fed mice mimic each of the histopathologic features of progression to colon cancer in humans that we tested. The features illustrated in Figures 6-11 include tubular adenomas, tubular adenomas with high grade dysplasia, sessile serrated adenomas, adenocarcinomas of category T1 (cancers that have invaded through the muscularis mucosa and extended into the submucosa), and adenocarcinomas of category T2 (cancers that have invaded through the submucosa and into the muscularis propria). As in the great majority of humans progressing to colon cancer, no tumors were found in the small intestines of these DOC-fed mice.

Locations of tumors in our mouse model and in humans

All of the tumors found in our previous study with male mice[9] were in the proximal colons of the mice. In our current study with female mice, the majority of tumors were in the proximal colon, with 44 of the 57 tumors or 77% of tumors being in the proximal colon. This is somewhat different from tumors in the human colon where tumors are found to be more nearly equally distributed between the proximal and distal regions of the colon. However, the level of DOC in the different regions of the human colon depends on two factors, while it was primarily dependent on only one factor in the mice fed diet + DOC. The first factor in humans is the continuous deconjugation and dehydroxylation (by bacteria) of the cholic acid entering the colon from the small intestine. This bacterial action generates newly formed DOC throughout the length of the colon[18]. The second factor in humans is the high level of absorption (about 50% overall) of DOC as it passes along all the regions of the colon[19]. In humans, the level of DOC would be about the same throughout the colon. In our mouse model, on the other hand, the level of DOC in mice fed diet + DOC starts off high in the proximal region of the colon. In contrast to humans, conversion of cholic acid to DOC is likely relatively insignificant for these mice since about 90% of the DOC in the colons of the mice fed diet + DOC comes from the added DOC in the diet rather than from conversion of cholic acid to DOC in the colon. Presumably, there is similar absorption of DOC from all regions of the colon in mice, as occurs in humans. Thus, there should be higher levels of DOC in the proximal regions of the colons of the mice compared to that in their distal regions. In our mice, much of the DOC would be absorbed as it travels down the length of the mouse colon. If tumors are caused by interaction of relatively high levels of DOC with colonic epithelial cells, then it is likely that, in our system, the majority of tumors would occur in the proximal colons of the mice, while in humans, with a more even distribution of DOC along the colon, tumors would occur in both the proximal and distal regions of the colon.

Tumors and colon cancers in mice occurred at an earlier age than normally found in humans. However, as reviewed by Cortopassi *et al*[20], multiple studies show that mice have about a 5.9-fold lower level of DNA repair than humans. A model proposed by these authors suggests that the earlier occurrence of colon cancer in mice fed diet + DOC, compared to humans, could be due to the DNA damaging nature of DOC and the lower DNA repair rate in mice.

Field defects in progression to cancer

Colon cancers are known to arise within a "field defect," an area of the colon predisposed to progression to cancer[21]. As pointed out by Rubin[22], field defects are of crucial importance in progression to cancer. Multiple tumors in a localized area during progression to colon cancer indicate a field defect.

Macroscopically, we found multiple colonic tumors in the same colonic area, indicating that colonic tumors in both mice and humans often occur within a field defect. We previously reported, by immunhistochemical evaluation, that the colonic mucosa surrounding human colon cancers has biomarker alterations indicative of a field defect as well[11]. We can speculate that some of the mutant or epigenetically altered cells are produced due to an early deficiency in ERCC1 (and possibly to deficiencies in other un-evaluated DNA repair proteins). Such cells would be genetically unstable and could acquire a growth advantage (*e.g.,* apoptosis resistance) due to further mutations and/or epimutations. We have shown that colonic epithelial cells grown in culture and repeatedly exposed to increasing concentrations of DOC underwent natural selection to develop resistance to apoptosis[23]. These apoptosis-resistant cells were altered in expression in 839 out of 5000 genes assessed by cDNA assay[23] and in 91 of 454 proteins detected by a proteomic analysis[24]. Cells with a growth advantage, upon proliferation, may form a defective field, which, with further mutation and epigenetic alteration due to bile acids, and further selection, could give rise to tumors, and eventually, to a colon cancer.

Oxidative DNA damage, the antioxidant CGA, and DNA repair in colon cancer

As reviewed by Bernstein *et al*[25] exposure of colon cells to high physiologic concentrations of DOC increases formation of reactive oxygen species (ROS), increases DNA damage, and causes apoptosis. A particularly important oxidative damage to DNA is 8-OH-dG, considered to play a central role in carcinogenesis[14]. A central enzyme in repair of oxidative damage to DNA is ERCC1[26]. In our present study 8-OH-dG is substantially increased and protein expression of ERCC1 is substantially decreased in the colonic epithelium of mice fed diet + DOC and progressing to colon cancer.

Chlorogenic acid (CGA) is an ester formed between caffeic acid and quinic acid, and is widely available in many food products, especially in coffee, blueberries and eggplant[27,28] and can even be purchased as diet supplement capsules containing 50% CGA. CGA is an excellent natural scavenger of free radicals because the one-elec-

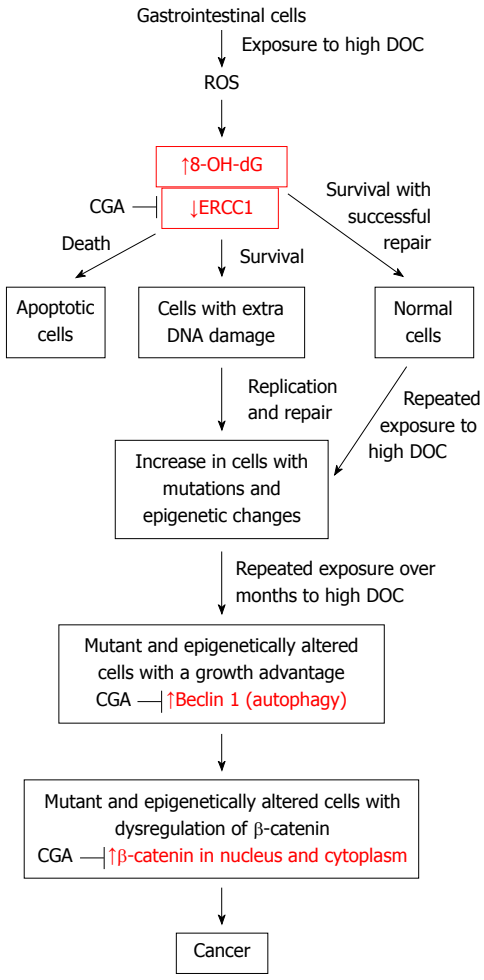

Figure 18 Likely path of progression to colon cancer in mice and humans, indicating key roles of the molecular markers evaluated here and the points of effects of chlorogenic acid in mice. CGA: Chlorogenic acid; DOC: Deoxycholic acid; ERCC1: Excision repair cross-complementation group 1.

tron oxidation product of CGA formed by the reaction with free radicals is rapidly broken down to products that cannot generate further free radicals[29].

We previously tested 19 antioxidants to evaluate their effect on expression of DNA repair proteins[30]. Only chlorogenic acid (CGA) and its metabolic derivatives increased expression of two DNA repair enzymes in that study. In our previous report on our new diet-related mouse model of colon cancer[9], CGA, fed to mice at a level equivalent to three cups of coffee a day for humans, substantially reduced the incidence of colon cancer for mice fed diet + DOC. Here, CGA in the diet largely prevented the reduction in protein expression of DNA repair protein ERCC1, central to repair of oxidative damage to DNA, that otherwise occurs with feeding mice diet + DOC.

Beclin-1 and autophagy

Beclin-1 is a central player in autophagy. The modulation of autophagy is now recognized as one of the hallmarks of human cancer cells. Accumulating evidence indicates that autophagy plays a role in the various stages of tumorigenesis. Depending on the type of cancer and the context, macroautophagy can be a tumor suppressor or it can help cancer cells to overcome metabolic stress and advance[15]. In particular, beclin-1 appears to be a central player in the mechanisms that control the level of p53. In addition, beclin-1 activates the autophagic pathway and this contributes to apoptosis resistance, which might have a role in carcinogenesis[31]. In mouse colonic epithelial tissues beclin-1 was increased in mice fed diet + DOC (Figure 15B), but this increase was reduced in mice fed diet + DOC + CGA (Figure 15C).

Beta-catenin in progression to cancer

Four major signaling pathways are frequently altered in the later stages of progression to sporadic human colon cancer, and three other pathways have also been identified. The most frequent pathways are Wnt/beta-catenin, TGF-beta receptor, Notch, and Hedgehog, while the other pathways are the EGFR, RAS/RAF/MAPK cascade and PI3K/Akt[32]. No one pathway is altered in all sporadic colon cancers. However, beta-catenin nuclear accumulation is found in 40% to 80% of primary human colon cancers[33,34] and in 67% of sessile serrated adenomas progressing towards human colon cancer[35]. We assessed beta-catenin and found that it is translocated into the nucleus of cells in the stem cell region of mouse colonic crypts in mice fed diet + DOC, but this translocation is reduced if CGA is also added to the diet (indicated in Figure 16).

Difficulties with previous rodent models of colon cancer

Rosenberg *et al*[36], in a 2009 review of then-current mouse models of colonic carcinogenesis, noted that they lack an invasive phenotype. Corpet *et al*[37] noted in 2005 that most then-current rodent models of colonic carcinogenesis did not share several significant genetic events and histopathological features of human colon cancers.

In the New Western diet (NWD)[38] mouse model of colon cancer (based on a diet deficient in calcium, vitamin D_3, fiber, methionine and choline, plus increased corn oil) mice developed the same frequency (4 out of 15 mice) of small intestinal tumors as colon tumors after 2 years on the diet. This is unlike intestinal cancers in humans where only 6% as many small intestinal cancers develop compared to the frequency of colon cancers[39]. In addition, no mice solely on the NWD developed fully invasive colonic adenocarcinomas[38].

Pathway of progression to colon cancer

A likely pathway for progression to colon cancer is shown in Figure 18. This figure indicates presumed major steps in progression to colon cancer. The key roles of the molecular markers we evaluated in our diet-related mouse model of colon cancer are shown in red. The effect of CGA on these markers is also indicated by arrows.

Bile acids, especially DOC, cause increases in DNA damaging ROS in colon cells[40-43]. DOC-induced ROS

are shown in Figure 18 as an early step in our diet-related pathway to colon cancer.

A major type of DNA damage caused by ROS is 8-OH-dG[44]. 8-OH-dG is mutagenic[45], and an initiator of carcinogenesis[14]. Thus, increased 8-OH-dG, as found by us in the epithelium of mice fed diet + DOC, is shown in Figure 18 as a key step in progression to colon cancer.

DNA damage appears to be a primary underlying cause of cancer[46]. Cells that retain unrepaired DNA damage, upon replication, may give rise to daughter cells with increased mutations by translesion synthesis[47,48]. Inaccurate or incomplete repair of DNA damages may also give rise to mutations or epigenetic alterations[49,50]. Such increased mutations and epigenetic alterations likely underlie progression to cancer, as indicated in Figure 18.

Deficiencies in DNA repair genes and genomic instability

In sporadic cancers, a deficiency in DNA repair may sometimes occur due to a mutation in a DNA repair gene. However, much more frequently, reduced or absent expression of DNA repair genes occurs due to epigenetic alterations that reduce or silence gene expression. For example, for 113 colorectal cancers examined in sequence, only four had a missense mutation in the DNA repair gene *MGMT*, while the majority had reduced MGMT protein expression due to methylation of the *MGMT* promoter region (an epigenetic alteration)[51]. Similarly, out of 119 cases of mismatch repair-deficient colorectal cancers that lacked DNA repair gene *PMS2* expression, PMS2 protein was deficient in 6 due to mutations in the *PMS2* gene, while in 103 cases PMS2 protein expression was deficient because its pairing partner the MLH1 protein was epigenetically repressed due to promoter methylation (PMS2 protein is unstable in the absence of MLH1 protein)[52]. In the other 10 cases, loss of PMS2 protein expression was likely due to epigenetic over-expression of the microRNA, miR-155, which down-regulates MLH1 protein expression[53]. Epigenetic deficiencies in expression of DNA repair proteins are virtually always present in colon cancers[46]. Epigenetically caused DNA repair protein deficiencies and the frequencies with which they are reported in colon cancers are MSH2 (13%), MLH1 (2%-65%), WRN (38%), MGMT (46%-90%), XPF (55%), PMS2 (88%) and ERCC1 (100%)[46]. ERCC1 protein deficiency was observed in all of the 47 human colon cancers evaluated[11] and thus ERCC1 deficiency appears to be one of the most prevalent DNA repair deficiencies in progression to colon cancer in humans. ERCC1 protein was also found to be deficient in histologically normal colonic epithelial tissues in mice fed diet + DOC and progressing to colon cancer (Figure 14).

A major characteristic of cancer is the presence of genomic instability (a mutator phenotype)[54]. This may be due to deficiency of a human DNA repair enzyme, such as ERCC1[11,46]. The average colon cancer has about 60 to 70 protein altering mutations of which about 3 or 4 may be "driver" mutations[55]. However, the protein coding part of the genome is only about 1.5% of the entire genome[56]. There are also about 20000 to 80000 mutations in the entire genome of various cancers[57,58]. This compares to the very low mutation frequency of about 70 new mutations in the entire genome between generations (parent to child) in humans[59,60]. The very high mutation frequency in cancer cells may be due to the frequent epigenetic deficiencies in DNA repair genes that likely occur early in progression to cancer. This is illustrated near the top of Figure 18. ERCC1 deficiency may have a major role in genomic instability in colon cancers. In our present study, mice progressing to colon cancer are deficient in protein expression of ERCC1 in the stem cell regions of colonic crypts.

The diet-related mouse model of colon cancer described here appears to be the closest model to human development of colon cancer that is currently available. It is based on elevated colonic levels of the natural endogenous bile acid DOC, long thought (from epidemiological evidence) to be important in initiation and progression to colon cancer[6,7]. It closely parallels human progression to colon cancer, both by the gold standard of histopathology and by the molecular markers tested. This mouse model may be uniquely useful in experiments involving the prevention or treatment of colon cancer.

COMMENTS

Background

Colon cancer is the second most frequent cause of cancer mortality among men and women combined, in both more developed and less developed areas of the world. Diet appears to be the major factor affecting frequency of colon cancer. Up to now, however, there has not been an established diet-related rodent model that closely parallels human progression to colon cancer. Such a model is needed to have an effective basis for experiments exploring the prevention or treatment of colon cancer.

Research frontiers

Bile acids delivered to the colon in response to a high fat diet have long been hypothesized to have a key role in development of colon cancer. In support of this hypothesis, it was recently found that the concentration of the bile acid deoxycholate in the feces of native Africans is only 1/5th as high as in African Americans, and the frequency of colorectal cancer in native Africans is less than 1/72nd the frequency of colorectal cancer in African Americans. An important area of research is to determine the molecular changes and neoplastic consequences caused by increased deoxycholate in the colon.

Innovations and breakthroughs

The study of experimental colon carcinogenesis in rodents has a long history, dating back about 70 years to an experiment of adding methylcholanthrene to the food of mice. Most studies were done with potent chemical carcinogens, which would not likely cause the same types of DNA damages that are caused by natural dietary factors. More recently, studies were also done with transgenic, knockout and knockin genetic models. In addition, a mouse model of colon cancer (based on a diet deficient in calcium, vitamin D_3, fiber, methionine and choline, plus increased corn oil) was devised. A notable disadvantage of these models was that induced tumors generally lacked an invasive and metastatic phenotype, and for many models, small intestinal neoplasias were often as frequent (or more frequent) than colon cancers, unlike the situation in humans. In addition, mutational alterations frequently present in human colon cancers were often not present in artificial rodent models of colon cancer. Thus, the finding that the natural endogenous bile acid deoxycholate actually caused colon cancer in a mouse model is an important contribution. Authors consider that this model should produce the typical types of DNA damages produced in humans by high physiologic levels of bile acids. Also, this model only produces cancers

in the colon, the location of almost all human intestinal cancers. Authors now show that the cancers produced are invasive and have morphological features and molecular markers consistent with those found in human progression to colon cancer.

Applications

The results of the present study indicate that this diet-related mouse model of colon cancer (with human physiologic levels of deoxycholate) will provide a more effective basis for experiments exploring the prevention or treatment of colon cancer than has previously been available.

Terminology

Human physiologic levels of deoxycholate are levels of deoxycholate found in humans eating a diet high in milk fat (sour cream, butter) and beef fat, or high in corn oil. Cancer mortality is the frequency of deaths due to a particular form of cancer.

Peer review

This study analyzes a novel diet-related model of colon cancer that parallels human progression to colon cancer, using both histomorphological criteria and molecular biomarkers. It also shows the ameliorating effects of dietary chlorogenic acid (a common component of blueberries, eggplant and apples) on molecular biomarkers of progression to colon cancer. This study is, undoubtedly, highly relevant for future research in human colonic cancer.

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ORIGINAL ARTICLE

Growth inhibition of colon cancer cells by compounds affecting AMPK activity

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Abstract

AIM: To determine if other molecules reported to modulate AMP-dependent protein kinase (AMPK) activity would have effects resembling those of metformin and phenformin on colon cancer cell proliferation and metabolism.

METHODS: Studies were performed with four human colon cancer cell lines, Caco-2, HCT116, HT29 and SW1116. The compounds that were studied included A-769662, 5-aminoimidazole-4-carboxamide-1-ribofuranoside, butyrate, (-)-epigallocatechin gallate (EGCG), KU-55933, quercetin, resveratrol and salicylates. The parameters that were measured were cell proliferation and viability, glucose uptake, lactate production and acidification of the incubation medium.

RESULTS: Investigations with several molecules that have been reported to be associated with AMPK activation (A-769662, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside, EGCG, KU-55933, quercetin, resveratrol and salicylates) or AMPK inhibition (compound C) failed to reveal increased medium acidification and increased glucose uptake in colon cancer cells as previously established with metformin and phenformin. The only exception was 5-aminosalicylic acid with which there were apparently lower glucose levels in the medium after incubation for 72 h. Further study in the absence of cells revealed that the effect was an artifact due to inhibition of the enzyme-linked glucose assay. The compounds were studied at concentrations that inhibited cell proliferation.

CONCLUSION: It was concluded that treatment with several agents that can affect AMPK activity resulted in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced, in contrast to the effect of biguanides.

Key words: Colon cancer cells; Proliferation; AMP-dependent protein kinase; Glucose metabolism

Core tip: Treatment with several agents that can affect AMP-dependent protein kinase activity resulted in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced, in contrast to the effect of biguanides.

Lea MA, Pourat J, Patel R, desBordes C. Growth inhibition of colon cancer cells by compounds affecting AMPK activity. *World J Gastrointest Oncol* 2014; 6(7): 244-252 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i7/244.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i7.244

INTRODUCTION

In previous publications we reported that the biguanides, metformin and phenformin, inhibited proliferation of

colon cancer cells under conditions in which glucose uptake was increased and there was increased glycolysis as judged by acidification of the incubation medium[1,2]. This is an unusual combination of effects and raises the question of whether other molecules might have similar action. Although the biguanides have a long history in the treatment of Type II diabetes there has been uncertainty regarding their mechanism of action[3,4]. Interest in mechanisms has been further stimulated by observations that metformin may exert a cancer chemopreventive effect and this has led to ongoing clinical trials against different types of cancer[5,6]. The most commonly suggested mechanisms for the action of biguanides include a stimulation of AMP-dependent protein kinase (AMPK) and inhibition of complex I in the mitochondrial electron transport chain. The hypothesis to be tested in the present work was that other molecules reported to modulate AMPK activity would have effects on colon cancer cell proliferation and metabolism resembling those of biguanides. We chose to examine the action of a variety of compounds that have been reported to activate or inhibit AMPK. Activators included A-769662[7], 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR)[8], (-)-epigallocatechin gallate (EGCG)[9], KU-55933[10], quercetin[11], resveratrol[12] and salicylates[13]. The most widely studied inhibitor of AMPK is compound C and that compound has been shown to affect proliferation of colon cancer cells[1]. Butyrate has been most commonly considered as an inhibitor of histone deacetylase activity but activation of AMPK by butyrate has been reported. In a previous study we observed that the induction of alkaline phosphatase by butyrate in colon cancer cells was not significantly affected by coincubation with A-769662[1]. However, in the present work some additive effects on metabolism and cell proliferation have been seen after coincubation of butyrate and A-76992 with colon cancer cells.

MATERIALS AND METHODS

Cells and determination of cell proliferation

SW1116, HCT116, HT29, and Caco-2 human colon cancer cells were obtained from the American Type Culture Collection, Rockville, MD, United States, and were incubated at 37 ℃ in RPMI-1640 medium with 5% fetal calf serum. Of these cell lines, the HCT116 cells exhibited the most rapid proliferation, and the slowest growth was seen with the SW1116 cells. Cell proliferation was generally monitored by the increase in protein. In studies with 96-well plates, the procedure involved staining with sulforhodamine B essentially as described by Vichai *et al*[14]. Cells were routinely allowed to attach to tissue culture dishes or 96-well plates for 24 h before changing the medium. The cells were then incubated for a further 72 h before determining the impact of the compounds under study on medium pH, glucose concentration, and cell proliferation as judged by protein mass. Cell viability was monitored using the Presto Blue Viability Reagent from Life Technologies Corporation, Carlsbad, CA, United States.

Reagents

A-769662 was purchased from LC Laboratories, Woburn, MA, United States. AICAR, butyrate, (-)-epigallocatechin gallate, metformin, phenformin, quercetin, resveratrol, salicylic acid, acetylsalicylic acid, 4-aminosalicylic acid and 5-aminosalicylic acid were obtained from Sigma-Aldrich, St. Louis, MO, United States. KU-55933 was purchased from Selleck Chemical, Houston, TX, United States.

pH determination

pH determination with an electrode was found previously to correlate well with changes in the light absorbance at 560 nm reflecting changes in the pH indicator, phenol red, where a higher absorbance reflects a higher pH[1]. The latter method was found particularly convenient for work with 96 well plates and was used routinely in the present work.

Glucose assay

Glucose was assayed in the cell culture medium using GAGO-20 Kit from Sigma-Aldrich. This is a colorimetric procedure in which the oxidation of glucose is coupled with glucose oxidase and peroxidase to the oxidation of dianisidine.

Lactate assay

Lactate in the medium was determined using the assay kit obtained from Eton Bioscience, San Diego, CA, United States.

Statistical analysis

Data are presented as means and standard deviations. Statistical significance of the results was determined by a two-tailed Student's *t* test or by Dunnett's test for multiple comparisons using the Instat program from GraphPad Software, Inc., La Jolla, CA, United States. A probability of less than 5% was considered significant and differences compared to the control are shown.

RESULTS

The uptake of glucose by HCT116 colon cancer cells was inhibited by incubation with butyrate or A-769662 for 72 h. This is shown in Figure 1A where the final glucose concentrations in the medium are shown after an initial glucose concentration of 2 mg/mL. Decreased glucose uptake paralleled decreased acidification of the incubation medium (Figure 1B) and decreased lactate production (Figure 1C). The data in Figure 1D indicate inhibitory effects of butyrate and A-769662 on proliferation of HCT116 cells as judged by staining with sulforhodamine B. The data in Figure 2A-D show similar responses in HT29 cells to those seen with HCT116 cells. Measurement of metabolic activity in HT29 cells as reflected in

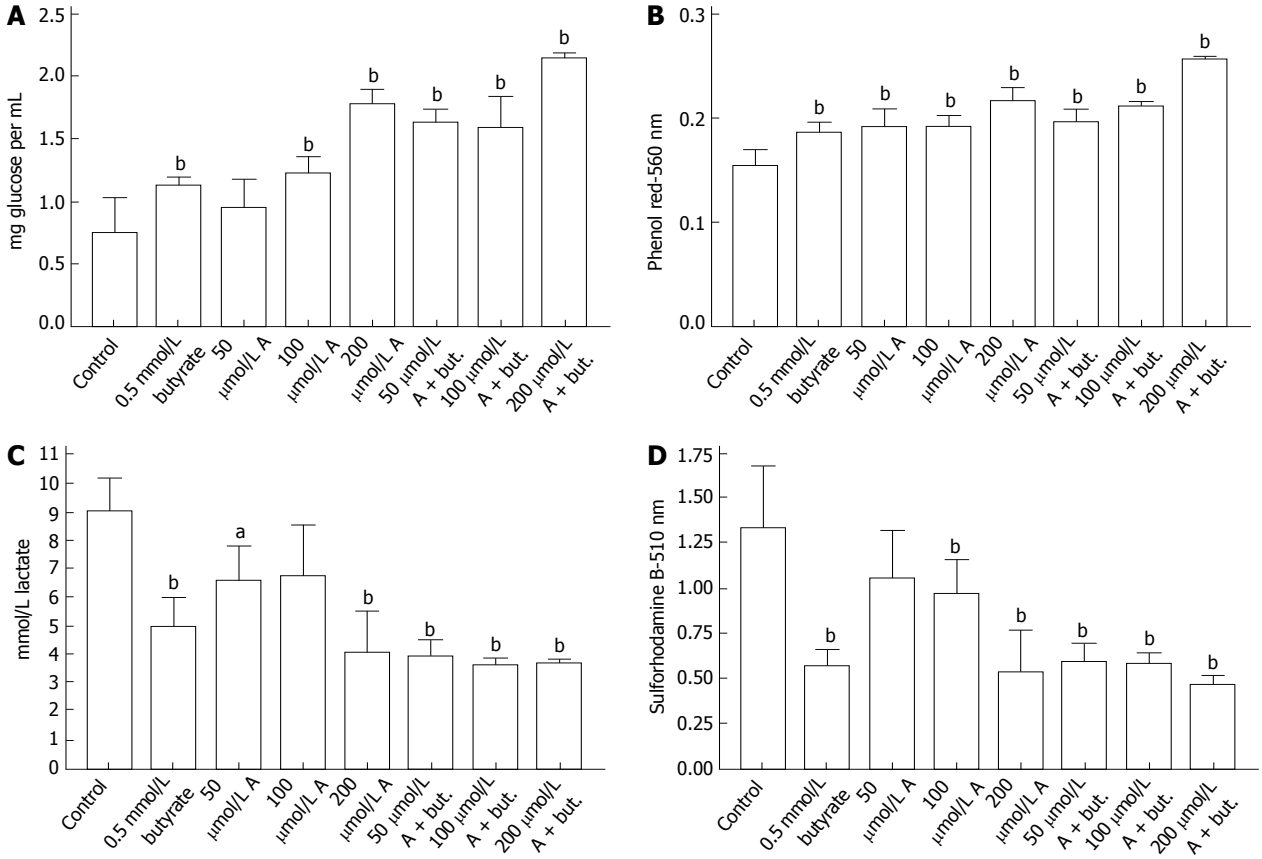
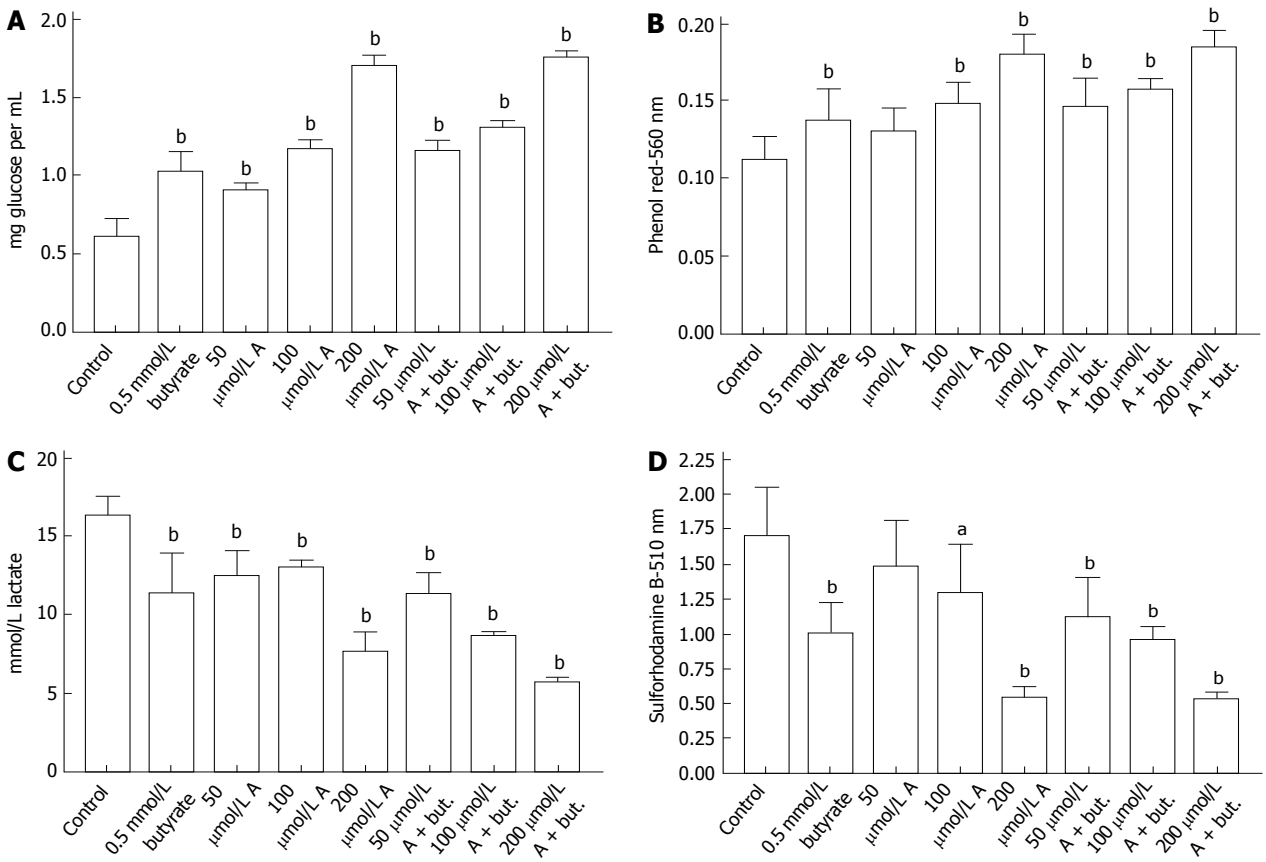


Figure 1 Effects of incubation of HCT116 WT cells for 72 h with butyrate. A-769662 (A) on glucose concentration of the incubation medium (A), medium pH (B), medium lactate concentration (C), and cell proliferation (D). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.



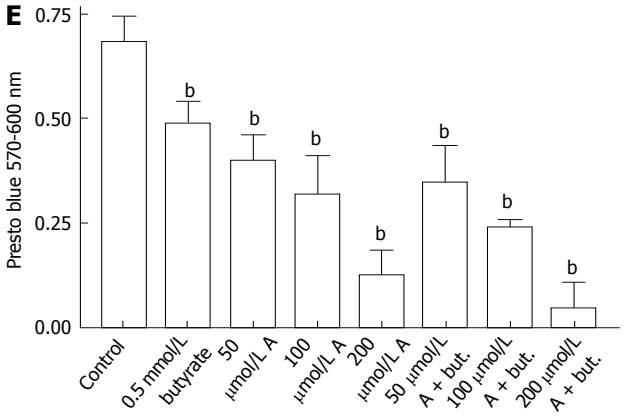


Figure 2 Effects of incubation of HT29 cells for 72 h with butyrate. A-769662 (A) on medium glucose concentration (A), medium pH (B), medium lactate concentration (C), cell proliferation (D) and reduction of Presto Blue (E). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

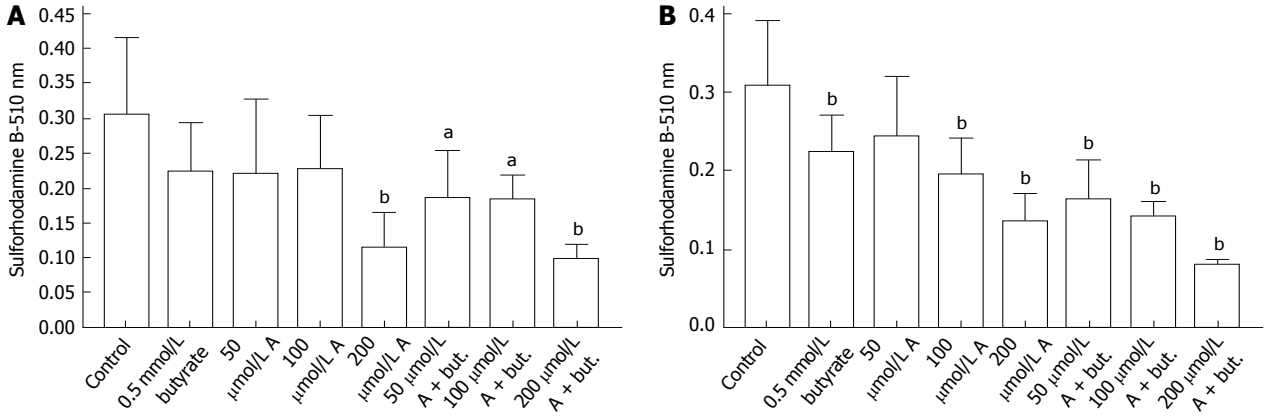


Figure 3 Effects of incubation of Caco-2 cells (A) and SW1116 cells (B) for 72 h with butyrate (but) and A-769662 (A) on cell proliferation. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

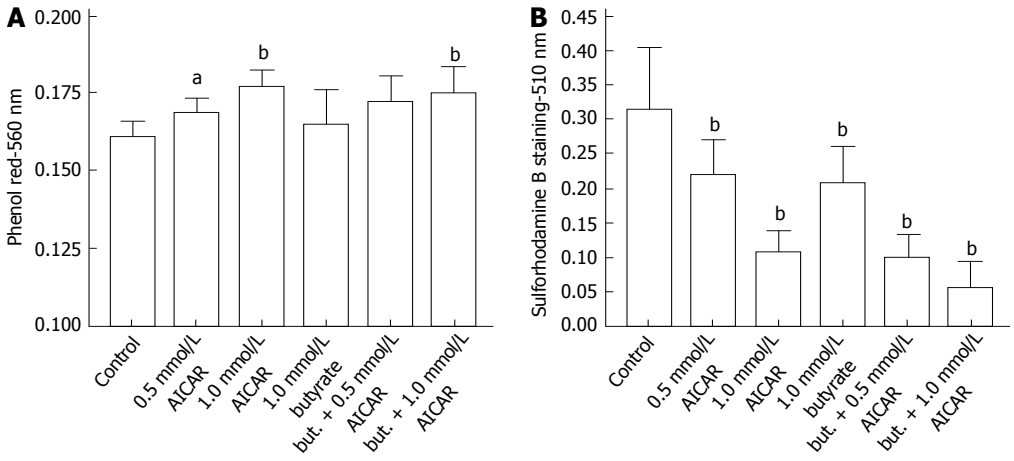


Figure 4 Effects of incubation of Caco-2 cells for 72 h with butyrate (but) and AICAR on medium acidification (A) and cell proliferation (B). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

the reduction of Presto Blue show a similar profile to that seen with sulforhodamine B staining and suggest some additive action when there is coincubation with butyrate and A-769662 (Figure 2E). Effects on metabolism in the more slowly growing Caco-2 and SW1116 cells were not as marked as in the more rapidly growing HT29 and HCT1116 cells but the results in Figure 3 show some degree of additive effect of butyrate and A76992 on the inhibition of cell proliferation.

Significant effects on glucose uptake were not seen when Caco-2 cells were incubated for 72 h with 0.5 and 1 mm AICAR but as shown in Figure 4A there were increases in medium pH suggesting less glycolysis and this was accompanied by decreased proliferation (Figure

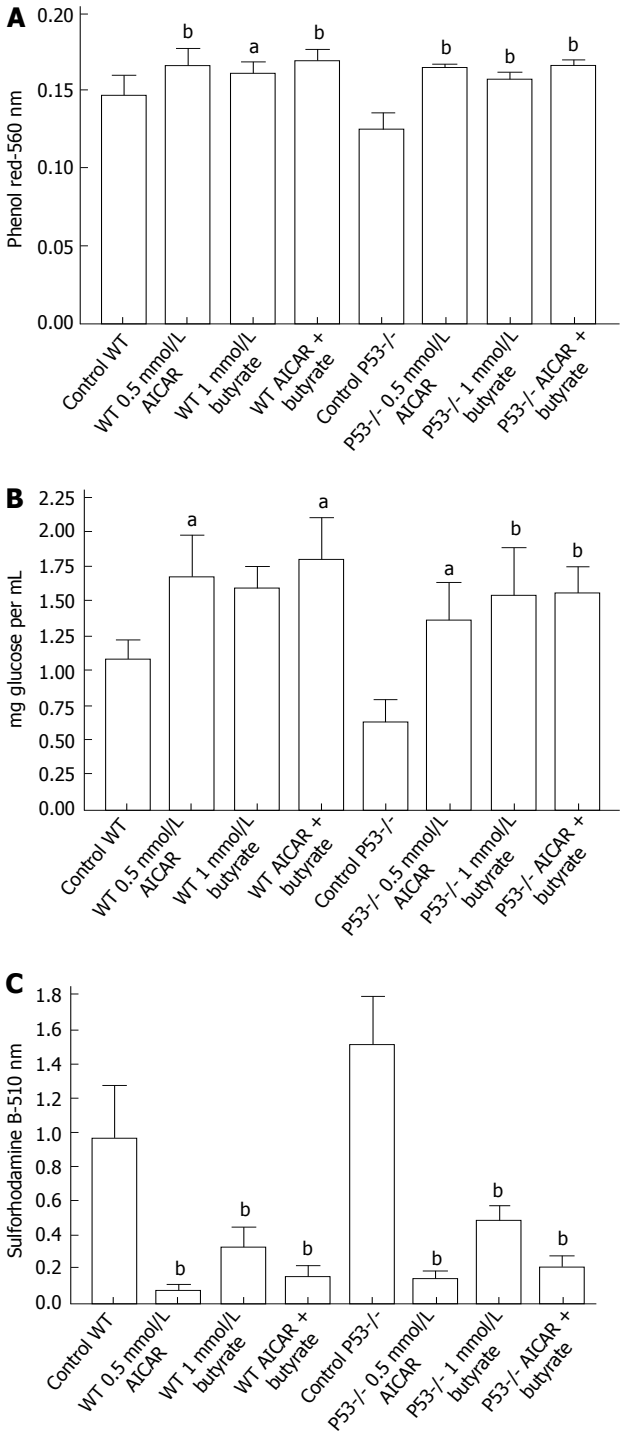


Figure 5 Effects of incubation of HCT116 wild type or p53 null cells. Effects of incubation of HCT116 wild type or p53 null cells for 72 h with butyrate and AICAR on acidification of the incubation medium (A), medium glucose concentration (B) and cell proliferation (C). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

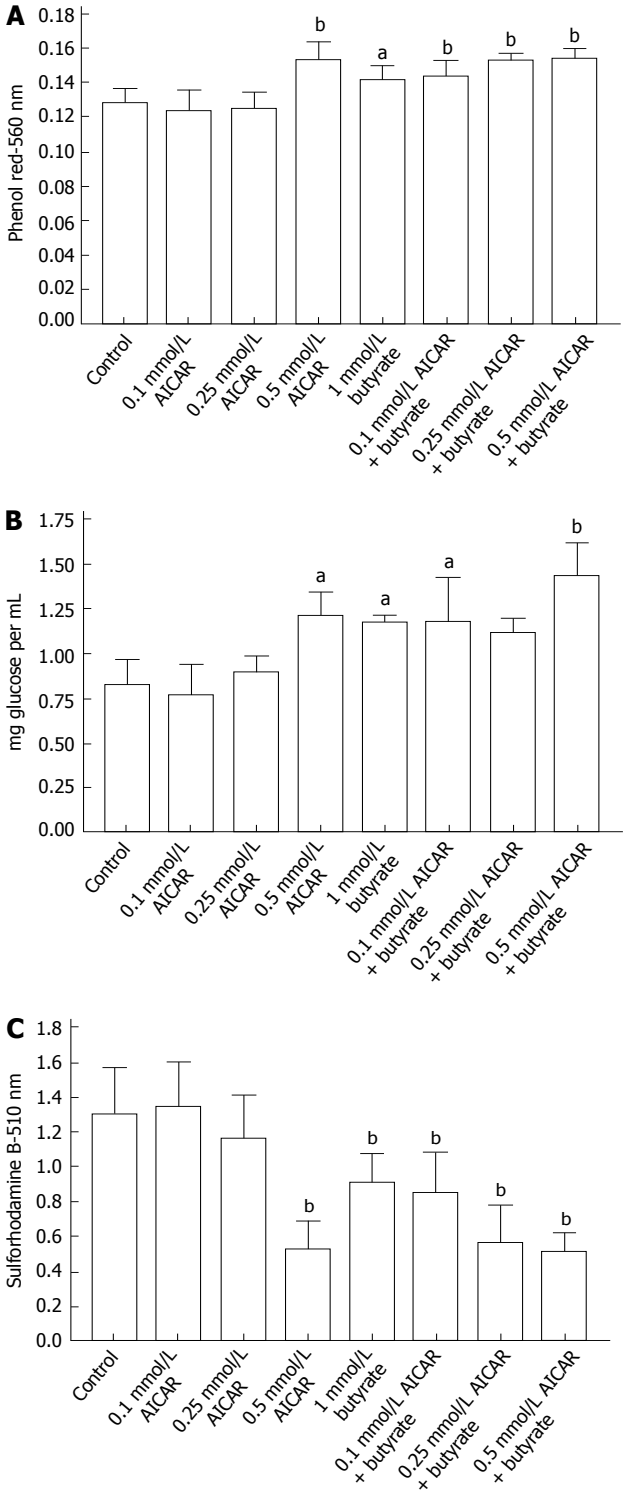


Figure 6 Effects of incubation of HT29 cells. Effects of incubation of HT29 cells for 72 h with butyrate and AICAR on acidification of the incubation medium (A), medium glucose concentration (B) and cell proliferation (C). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

4B). With the more rapidly dividing HCT116 wild type or p53 null cells there were significant decreases in medium acidification when cells were incubated with 0.5 mmol/L AICAR (Figure 5A) together with decreased glucose uptake (Figure 5B) and decreased cell proliferation (Figure 5C). Similarly with HT29 cells, AICAR at 0.1, 0.25 and 0.5 mmol/L caused the same trends (Figure 6).

In addition to effects of AMPK activators, inhibitory effects on medium acidification, glucose uptake, lactate production, reduction of Presto Blue and cell proliferation were also seen when the AMPK inhibitor, compound C, was incubated for 72 h with HCT116 cells (Figure 7).

Studies with several molecules that have been re-

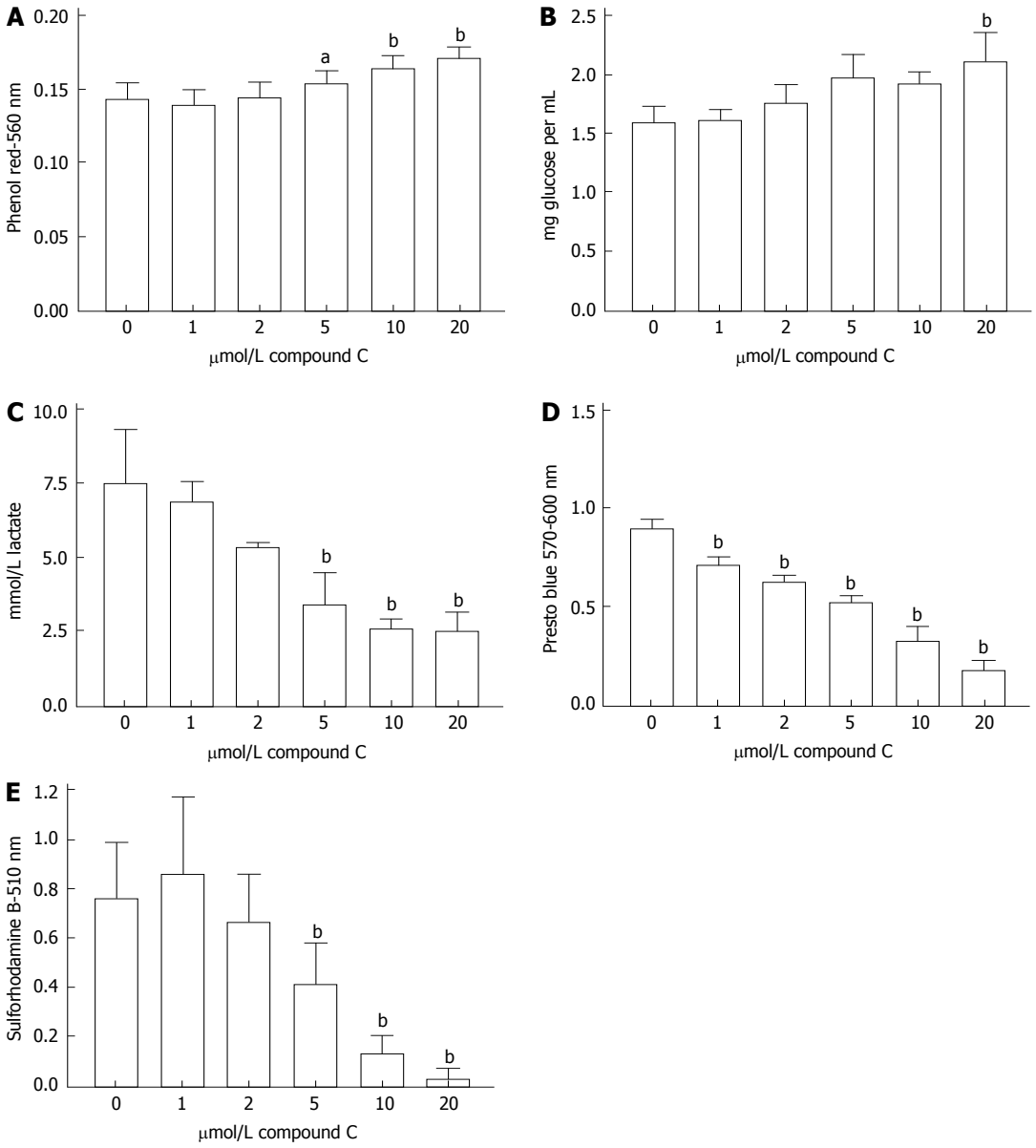


Figure 7 Effects of incubation of HCT116 WT cells. Effects of incubation of HCT116 WT cells for 72 h with compound C on acidification of the incubation medium (A), glucose concentration of the incubation medium (B), medium lactate concentration (C), reduction of Presto blue (D) and cell proliferation (E). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

ported to be associated with AMPK activation (salicylates, EGCG, KU-55933, quercetin and resveratrol) failed to reveal increased medium acidification and increased glucose uptake in colon cancer cells as previously established with metformin and phenformin[1]. The only exception was 5-aminosalicylic acid with which there were apparently lower glucose levels in the medium after incubation for 72 h (Figure 8A). This was surprising because the effect was not associated with increased medium acidification as seen with the biguanides and was seen at a concentration that did not result in significant inhibition of cell proliferation (Figure 8B). Further examination in the absence of cells revealed that the effect was an artifact due to inhibition of the enzyme-linked glucose assay. There was specificity for the effect because it was seen with 5-aminosalicylic acid but not with 4-aminosalicylic acid (Figure 9). The effect was seen with two samples of 5-aminosalicylic acid from Sigma-Aldrich, one containing 95% and the other containing 98% of the compound.

DISCUSSION

Our previous studies on the effects of metformin and phenformin on colon cancer cells revealed an unusual combination of effects[1]. These were an inhibition of cell proliferation despite an increase in glucose uptake and an increase in lactate production as monitored by acidification of the medium. Information in the literature suggests that biguanides may inhibit complex 1 of the mitochondrial transport chain and may result in activation of AMPK. The latter effect may not be direct and may be a consequence of increased levels of AMP and ADP or may be mediated through an upstream kinase, LKB1. We chose to examine the significance of AMPK activation on

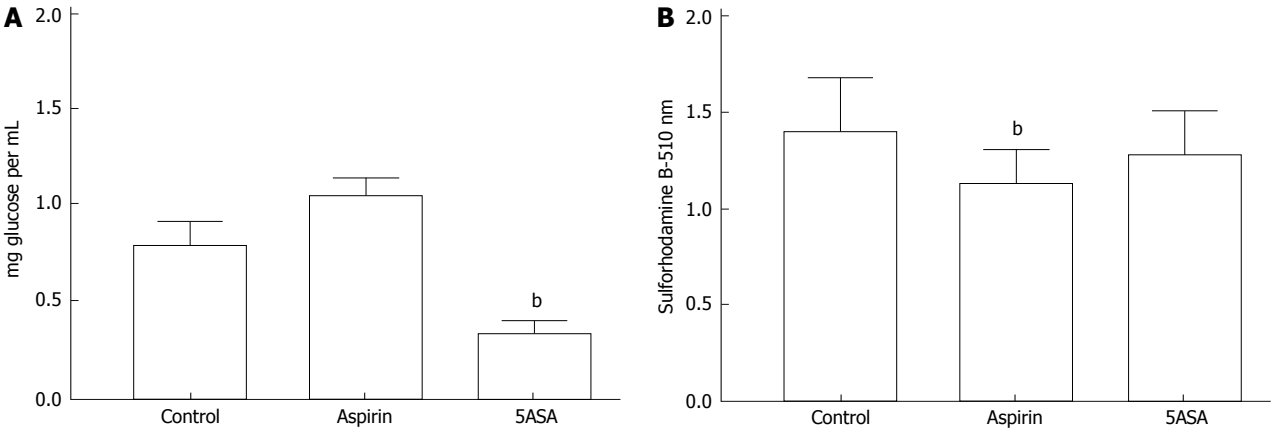


Figure 8 Effect of incubation of HT29 cells. Effect of incubation of HT29 cells for 72 h with 1 mmol/L acetylsalicylic acid (aspirin) and 1 mmol/L 5-aminosalicylic acid (5ASA) on glucose concentration of the incubation medium (A) and cell proliferation (B). $^{b}P < 0.01$ *vs* control group.

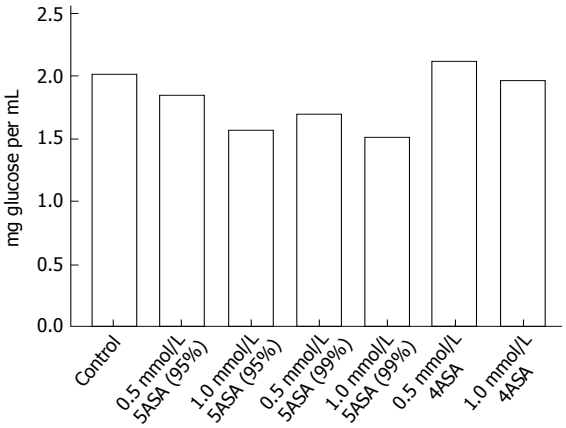


Figure 9 Effect of 5-aminosalicylic acid and 4-aminosalicylic acid. Effect of 5-aminosalicylic acid (5ASA) and 4-aminosalicylic acid (4ASA) on the assay of glucose in the RPMI 1640 medium that had not been incubated with colon cancer cells.

the metabolism and proliferation of colon cancer cells by studying the action of a variety of compounds reported to affect AMPK activity. The best characterized of these are A-769662[15] and AICAR[16]. These two compounds were found to be potential inhibitors of colon cancer cell proliferation but we observed neither an increase in glucose uptake nor increased medium acidification. To the contrary, decreased glucose uptake and decreased medium acidification was seen particularly with the more rapidly proliferating HT29 and HCT116 colon cancer cells.

Some of the compounds that were studied have been reported to be activators or inhibitors in different systems. Thus, quercetin has been reported to be an activator of AMPK[11] but Kim *et al*[17] found inhibition of AMPK by quercetin in HCT116 cells. Activation of AMPK by resveratrol has been reported[12,18] but Skrobuk *et al*[19] reported a situation in skeletal muscle where AMPK was inhibited by resveratrol. Compound C has consistently been found to be an inhibitor of AMPK. Although Compound C is a cell-permeable pyrrazolopyrimidine compound that can act as a reversible and ATP-competitive inhibitor of AMPK, actions on other target molecules have been reported[20]. We have extended our earlier studies with compound C and found that at concentrations frequently used to inhibit AMPK (1-10 μmol/L) it can be a potent inhibitor of colon cancer cell proliferation, most notably with HCT116 cells. Under those circumstances there was decreased glucose uptake and decreased acidification of the medium.

Potential chemopreventive action against colon cancer has been noted for some salicylates including acetylsalicylate[21] and 5-aminosalicylate[22]. At a concentration of 1 mmol/L we found that acetylsalicylic acid was a more potent inhibitor of colon cancer cell proliferation than 5-aminosalicylic acid. However, only with 5-aminosalicylic acid was there an apparent increase in glucose uptake. Further studies in the absence of cells indicated that this effect was due to interference with the enzyme-linked assay procedure for glucose. The assay uses a combination of glucose oxidase and peroxidase. It remains to be established whether one of these enzymes was more sensitive to the action of 5-aminosalicylic acid.

The tendency of cancer cells to show increased rates of glucose uptake and glycolysis even under aerobic conditions has become known as the Warburg effect. There is a paradox in that biguanides are of interest for their preventive or therapeutic action against cancer despite the observation that they seem to enhance the Warburg effect. The degree to which activation of AMPK relates to anti-cancer actions of biguanides remains an area of uncertainty[23-25]. It may be concluded from the present study that treatment with several agents that can affect AMPK activity results in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced.

COMMENTS

Background

Although there is a long history of the use of biguanides such as metformin in the treatment of type 2 diabetes, there is recent interest in the potential value of biguanides in the prevention and therapy of cancer. Rationale use of biguanides will be aided by comparison of their action with other compounds that can also

affect AMP-dependent protein kinase (AMPK) activity.

Research frontiers

Ongoing studies are investigating whether actions of biguanides on cancer cells relate to modulation of AMPK activity, effects mediated through inhibition of mitochondrial electron transport or changes in circulating insulin levels or combinations of these actions.

Innovations and breakthroughs

The observations described here emphasize that while biguanides and other compounds that modulate AMPK activity can affect the proliferation of cancer cells, there appears to be a unique pattern in the effects of metformin and phenformin that is also associated with increased glucose uptake and acidification of the extracellular environment.

Applications

The present work adds to the authors' knowledge of combined action of biguanides with other agents that may guide future combination therapies for the treatment of colon cancer. The results emphasize the need to better characterize actions of biguanides that relate to mechanisms other than modulation of AMPK activity.

Terminology

AMPK regulates metabolism so as to increase ATP production and limit ATP utilization. One potential mechanism of action for biguanides is to cause the upregulation of AMPK.

Peer review

The authors have investigated the inhibition of growth of colon cancer cells by compounds that affect AMPK activity but have divergent effect on metabolism. They have managed to show that treatment with several agents that can affect AMPK activity results in the inhibition of the proliferation of colon cancer cells under conditions in which glucose metabolism is not enhanced.

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RETROSPECTIVE STUDY

Prevalence and clinicopathological characteristics of appendiceal carcinoids in Sharjah (United Arab Emirates)

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Abstract

AIM: To determine the incidence and clinico-pathological profile of appendiceal carcinoids in a cohort of patients undergoing emergency appendicectomies for clinically suspected acute appendicitis in Sharjah, United Arab Emirates (UAE).

METHODS: The study included the retrospective data of 964 patients operated for clinically suspected acute appendicitis, and the resected specimens were received at Al-Qasmi Hospital (Sharjah) from January 2010 to December 2010. The data of the patients who were histologically reported to have carcinoid tumors of the appendix were extensively evaluated for the patient's demographics, indication for surgery, surgical procedure, tumor localization in the appendix, diameter of the lesion, concomitant appendicitis, immunohistochemistry studies and clinical follow-up.

RESULTS: Out of the 964 patients included in the study, 9 (0.93%) were found to have appendiceal carcinoids. The mean age reported was 28.7 years with a male to female ratio of 2:1. Eight tumors were located near the tip of the appendix with a mean diameter of 3.3 mm, while the remaining one was near the proximal end of the appendix. All the cases were associated with concomitant suppurative appendicitis. In seven reported cases, tumors were confined to the muscular layer while in one case each there was an extension to the serosa and mesoappendix, respectively. All tumors were found to be positive for chromogranin A, synaptophysin and neuron-specific enolase on immunohistochemistry but negative for cytokeratin-7. None of the patients developed recurrence or any reportable complications in the short follow-up period (12-26 mo) that was arranged as a six-monthly re-evaluation by abdominal ultrasonography.

CONCLUSION: Our study found a higher incidence of appendiceal carcinoids in patients undergoing emergency appendectomy for acute appendicitis in Sharjah, UAE compared to two previous studies from the Persian Gulf region. Interestingly, tumors were found to be more commonly in young males, which is in contrast to previous studies. Moreover, all the tumors were positive for common neuroendocrine markers.

Key words: Appendix; Carcinoid; Prevalence; Sharjah United Arab Emirates

Core tip: Incidence of appendiceal carcinoids is higher in patients undergoing emergency appendectomy for acute appendicitis in Emirate of Sharjah compared to two previous studies from the same geographical region. Moreover, tumors were found more commonly in

young males in contrary to previous studies.

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INTRODUCTION

Carcinoid tumors are rare, slow-growing neuroendocrine tumors arising from the enterochromaffin cells disseminated throughout the gastrointestinal and bronchopulmonary systems[1]. The biological behavior of these tumors is poorly understood. Carcinoid tumors are considered indolent tumors as compared to adenocarcinoma, yet they have a potential to exhibit highly aggressive behavior. Although in 2004 they accounted for 1.25% of all malignancies, their frequency is augmenting by 6% annually[2]. In an American study the most common primary tumor site varied by race, with the lung being the most common in white patients, and the rectum as the most common site in Asian/Pacific Islander, American Indian/Alaskan Native, and African American patients[3].

The incidence of gastrointestinal carcinoids in both males and females has concurrently increased. A recent study from England analyzing the anatomic distribution of the tumors in 10324 cases revealed the commonest site to be the appendix, small intestine, colon, stomach and rectum in the decreasing order of frequency[4]. Additionally, the largest absolute increase in incidence of the carcinoid was also reported at the site of the appendix[4]. Recent data report the overall incidence of carcinoid tumors among patients undergoing emergency appendectomies between 0.27% and 1.6%[5,6].

Appendiceal carcinoid tumors are clinically silent and are usually an incidental finding in patients undergoing surgery for suspected acute appendicitis or during incidental appendectomy in the course of relevant abdominal surgery procedures[7]. Most appendiceal carcinoids are located at the tip of the organ. They are usually diminutive, measuring less than 1 cm, and rarely grow beyond than 2 cm in diameter[8]. Immunohistochemically carcinoid tumors of the gastrointestinal tract including the appendix express general neuroendocrine markers, such as chromogranin A, synaptophysin, non-specific enolase (NSE), CD56 and glucagon[9]. The gold standard treatment is surgical treatment by resection of the whole appendix for carcinoids located around the tip. In cases where the tumor is larger than 2 cm or located at the base of the appendix, a wider resection has to be performed with right hemicolectomy[1,2,4] .

The aim of the current study was to determine the incidence and clinicopathological characteristics of appendiceal carcinoids along with their immunohistochemical profile in a cohort of patients undergoing emergency appendectomies for clinically suspected acute appendicitis in Sharjah, United Arab Emirates (UAE).

Table 1 Clinicopathological characteristics of patients with appendiceal carcinoids from Emirates of Sharjah

Patient number[1]	Age (yr)	Gender	Tumor size (mm)	Extension[2]	Tumor localization
1	25	M	8	Serosal layer	28 mm from proximal end
2	29	M	4	Mesoappendix	Tip
3	33	M	4	Muscular layer	Tip
4	19	M	2	Muscular layer	2 mm from tip
5	28	M	1	Muscular layer	Tip
6	54	M	1	Muscular layer	6 mm from tip
7	25	F	4	Muscular layer	13 mm from tip
8	18	F	3	Muscular layer	Tip
9	27	F	3	Muscular layer	10 mm from tip

[1]All cases underwent open appendectomy for clinical diagnosis of appendicitis which was further confirmed on microscopic examination; [2]No vascular invasion was identified in any case. M: Male; F: Female.

MATERIALS AND METHODS

This retrospective study was carried out at the Pathology Department of Al-Qasmi Hospital, Sharjah, UAE, which is the only tertiary care government facility in the region for the histopathological analysis of the surgical specimens. This study includes all consecutive patients who underwent appendectomies between January 2010 and December 2010 in Sharjah, UAE, and their specimens were received at the hospital for analysis. Only the data of the patients who were histologically reported to have carcinoid tumors of the appendix was reviewed for the patient's age, gender, indication for surgery and surgical procedure. The histological analysis included tumor localization in the appendix, evaluation of the diameter of the lesion after fixation with formaldehyde, concomitant appendicitis, and immunohistochemical analysis of chromograninin A, synaptophysin, NSE, serotonin, carcinoembryonic antigen (CEA), CK-7 and cytokeratin-20 (CK-20). Patient follow-up was conducted for those diagnosed with carcinoids only every 6 mo and recurrence evaluated by abdominal ultrasonography.

RESULTS

Nine hundred and sixty-four patients underwent appendectomies during the study period, of whom 9 (0.93%) were found to have histological evidence of carcinoid tumors of the appendix. The clinicopathological data in relation to carcinoids are shown in Table 1. There were 6 male and 3 female patients with a mean age of 28.7 years (range, 18-54 years). All the cases were operated for a clinical suspicion of appendicitis. Histologically 4 carcinoid lesions were demonstrated at the tip, another 4 ranged from 2-13 mm away from the tip and one lesion was located 28 mm from the base of the appendix. The

Table 2 Immunohistochemical characterization of appendiceal carcinoid tumors in patients from Emirates of Sharjah

Patient number	Age (yr)	Sex	CG	Synaptophysin	NSE	5-HT	CEA	CK20	CK7
1	25	M	+	+	+	-	-	-	-
2	29	M	+	+	+	+	-	-	-
3	33	M	+	+	+	-	-	-	-
4	19	M	+	+	+	-	-	+	-
5	28	M	N/D	N/D	N/D	N/D	N/D	N/D	N/D
6	54	M	+	+	+	+	+	-	-
7	25	F	+	+	+	+	-	-	-
8	18	F	+	+	+	-	-	-	-
9	27	F	+	+	+	+	-	-	-

CG: Chromogranin; NSE: Non-specific enolase; 5-HT: Serotonin; CEA: Carcinoembryonic antigen; CK-20: Cytokeratin 20; CK-7: Cytokeratin 7; N/D: Not determined as the tissue sample was unavailable for the staining procedure; M: Male; F: Female.

mean diameter of the tumors was 3.3 mm (range, 1-8 mm). Concomitant suppurative appendicitis was present in all cases. Seven tumors were confined to the muscular layer, while one case exhibited an extension to the serosa and another extended to the mesoappendix. The margins of all the resected tissue samples received for histological analysis, however, were free of tumor cells.

In one case the tissue sample from the tip was very infinitesimal to be evaluated by immunohistochemistry (IHC). The rest eight tumors were positive for chromogranin A, synaptophysin and NSE as shown in Table 2. Four tumors were additionally found to be positive for serotonin and one each for CEA and CK-20. None of the tumors was positive for CK-7.

All patients remained disease-free after a median follow-up duration of 22 mo (range, 12-26 mo).

DISCUSSION

Carcinoid tumors were not considered to be common tumors, but recent studies suggest an abrupt increase in their incidence and prevalence over the last few decades. Additionally, the appendix has been identified as one of the most common sites for carcinoids in the gastrointestinal tract[3,10]. The reason for this rise remains, as yet, obscure, although an increase in the number of elective appendectomies was considered to be one of the contributing factors. Contrary to this belief, a recent study demonstrated that the number of surgeries did not actually influence the incidence of appendiceal carcinoids[6]. However, more extensive pathological examination including multiple sections from different parts of the appendix may have played a part in detecting even the tiny foci of the tumors. Our present findings validate this hypothesis since most of the carcinoids identified were relatively small in size (1-4 mm in diameter). Carcinoid tumors are generally asymptomatic due to their small size and specific location in the appendix and are commonly diagnosed as an incidental finding in emergency or elective appendectomy specimens[11]. Although the majority of the carcinoids exhibit benign behavior, they do have a malignant potential with the ability to metastasize[7].

Our present study reports the incidence of carcinoid tumors at 0.93% per annum in the pathological specimens obtained during emergency appendectomies. This incidence is quite high compared to that reported by two other studies conducted in the same geographical region. The reported incidence in appendectomy specimens from Iran was 0.2% and that from Saudi Arabia 0.6%[12,13]. However, in most studies from other geographical regions the incidental histological diagnosis of carcinoid ranged from 0.3%-0.9% in patients undergoing appendectomy[8]. In a recent study conducted in a community teaching hospital in South Australia, appendiceal carcinoids were even found to occur in 1.6% of emergency appendectomies performed for acute appendicitis[6].

We did not observe a female preponderance in our patients with carcinoids as suggested in many previous studies[12-14]. We are unable to explain this gender disparity in our study where males were affected by this neoplastic lesion twice as frequently as females. There may be, however, a strong environmental bias in the UAE for this discrepancy. The gut microbiome influences both the development of the mucosal immune system as well as the regulation of epithelial regeneration[15]. Previous literature has indicated carcinoid tumors to be distributed among younger age groups (20-30 years of age) and their preferential location in the tip of the appendix, with the latter being attributed to the increased density of subepithelial neuroendocrine cells near the tip[16,17]. Our observations in the present study confirm these findings (Table 1). The average age for males was 31.3 years while for females it was 23.3 years. The mean overall age of the patients was 28.7 years.

Approximately 80% of appendiceal carcinoids are less than one centimeter in diameter[8]. Our present findings are consistent with previous studies as the tumor size in all cases in our study were less than one centimeter, with eight cases measuring between 1 and 5 mm and one 8 mm in diameter. Seven carcinoids were confined to the muscular layer, while one extended into the serosal layer and another one was located in the mesoappendix (Table 1).

All carcinoid tumors evaluated in this series showed positive IHC staining for common neuroendocrine markers. Interestingly, all the samples identified were positive for chromogranin A, synaptophysin and neu-

ron-specific enolase (Table 2). However, four carcinoids were positive for serotonin and one each for CEA and CK-20, respectively, all of them had a size between 1-4 mm. A previous study has demonstrated variable staining for these markers (62%-85%) in gastrointestinal carcinoinds[9]. The staining characteristics observed in our study were not associated with any other clinicopathological characteristics.

Although some carcinoids have been reported to be aggressive, none of the patients had recurrence or any reportable complications in the short follow-up period (12-26 mo). Histological analysis of the draining lymph nodes or the liver was not performed due to gross normal appearance and unremarkable abdominal ultrasonographic findings in these patients. The metastatic potential of carcinoids cannot be accurately assessed based on the follow-up duration, and this is a limitation of the current study.

Our seminal study from this region shows the incidence of appendiceal carcinoids in patients undergoing emergency appendectomies for clinically suspected acute appendicitis from Sharjah, UAE to be higher than that reported by two previous studies from the same geographical region. Contrary to other studies, young males were involved two times more commonly than the females. All tumors were found positive for common neuroendocrine markers.

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COMMENTS

Background

Carcinoid tumors are considered to be one of the commonest tumors in the appendix. Their incidence has been shown to vary in different studies and this seminal study details the prevalence of these tumors in the United Arab Emirates.

Innovations and breakthroughs

This is the first study from the region that shows that the incidence of appendiceal carcinoid tumors has augmented as compared to the previous studies from the region. Interestingly, this rise is observed in the young male population instead of the females, as highlighted in previous studies.

Applications

Such a difference in incidence necessitates an investigative research into the etiology and further monitoring to evaluate the trend of these tumors that may be associated with environmental factors due to changes in the gut microbiome. Repetitive evaluations are fundamental to assess incidence rates in cancer demographics. In addition data from other countries in the Persian Gulf region can provide a better global perspective.

Peer review

The authors present a subject of importance for the surgical community: the carcinoids of the appendix.

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PROSPECTIVE STUDY

Patient prompting of their physician resulted in increased colon cancer screening referrals

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Abstract

AIM: To determine whether a communication instrument provided to patients prior to their primary care physician (PCP) visit initiates a conversation with their PCP about colorectal cancer screening (CRC-S), impacting screening referral rates in fully insured and underinsured patients.

METHODS: A prospective randomized control study was performed at a single academic center outpatient internal medicine (IRMC, underinsured) and family medicine (FMRC, insured) resident clinics prior to scheduled visits. In the intervention group, a pamphlet about the benefit of CRC-S and a reminder card were given to patients before the scheduled visit for prompting of CRC-S referral by their PCP. The main outcome measured was frequency of CRC-S referral in each clinic after intervention.

RESULTS: In the IRMC, 148 patients participated, a control group of 72 patients (40F and 32M) and 76 patients (48F and 28M) in the intervention group. Referrals for CRC-S occurred in 45/72 (63%) of control *vs* 70/76 (92%) in the intervention group ($P \leqslant 0.001$). In the FMRC, 126 patients participated, 66 (39F:27M) control and 60 (33F:27M) in the intervention group. CRC-S referrals occurred in 47/66 (71%) of controls *vs* 56/60 (98%) in the intervention group ($P \leqslant 0.001$).

CONCLUSION: Patient initiated physician prompting produced a significant referral increase for CRC-S in underinsured and insured patient populations. Additional investigation aimed at increasing CRC-S acceptance is warranted.

Key words: Colon cancer; Screening; Primary care; Physician patient relationship; Referral

Core tip: Colon cancer screening only performed in approximately 60% of Americans over 50 years old. Inadequate communication between patient and physician is a significant obstacle to obtaining appropriate screening, especially in the underinsured population. Patient initiated prompting of their primary care physician for colorectal cancer screening with colonoscopy increased referrals in both underinsured and insured patient groups.

Le V, Syed S, Vega KJ, Sharma T, Madhoun MF, Srinivasan N, Houchen CW. Patient prompting of their physician resulted in increased colon cancer screening referrals. *World J Gastrointest Oncol* 2014; 6(7): 257-262 Available from: URL: http://www.wjgnet.com/1948-5204/full/v6/i7/257.htm DOI: http://dx.doi.org/10.4251/wjgo.v6.i7.257

INTRODUCTION

In spite of the available evidence suggesting effectiveness of colorectal cancer screening (CRC-S), approximately 50% of the United States population over 50 years old has not had CRC-S[1]. According to the National Cancer Institute, in 2009 the estimated new cases of colon cancer and rectal cancer in United States were 106100 and 40870 respectively. The estimated death of these combined cancers was 49920 (www.cancer.gov). Several studies have been conducted to understand the barriers for colorectal screening[2]. Inadequate communication between the primary care physician (PCP) and patient, including lack of a physician's recommendation for testing and patients unawareness were found to be important barriers[2-4]. Other investigators have shown colonoscopy as a safe and feasible primary screening test[5]. In addition, studies have also shown that in average risk patients, colonoscopy screening found 0.5%-1.0% have colon cancers and 5%-10% have advanced neoplasia that can be removed during the screening[5-9]. Providing educational material and a method for the patient to express interest in CRC-S to their PCP could increase referral for this screening. The aim of our study was to determine if patient initiated prompting of their PCP for CRC-S would increase referrals in both underinsured and insured patients.

MATERIALS AND METHODS

From November 2008 to November 2010, all patients seen in Family Medicine Resident Clinic (FMRC, insured) and Internal Medicine Resident Clinic (IMRC, underinsured) waiting areas were screened for CRC-S eligibility. Those patients meeting criteria for screening but never having been screened previously were considered eligible for the study. Eligible patients were assigned randomly to either a control or intervention group. Intervention consisted of a pamphlet describing the benefit of CRC-S, given to patients prior to their PCP visit and a reminder note about CRC screening to be given to their physician during the encounter. The pamphlet discussed colon cancer incidence, frequency, deaths, prevention, need for screening, risk factors, symptoms, available screening methods with colonoscopy preferred based on ACG guidelines. In order to not reveal the purpose of our study to resident physicians, patients were randomly assigned as control group or intervention group on different clinic days. Since, each resident physician only see patients on one specific day of clinic, and by randomizing patients on the same day will allow the physicians to figure out our study if he received a reminder note on one patient and not the other. A two-page questionnaire was designed to assess the referral patterns and preferred screening method for CRC. Questions on the survey included demographic parameters (age, race, gender, and education level), whether their PCP had referred them for CRC-S, the screening method recommended, whether the participants accepted the screening referral, presence of insurance coverage for CRC-S, and knowledge that CRC could be prevented using screening. Upon completion of the study, all patients in the control group were given the CRC-S pamphlet for use.

The primary outcome was to determine if patient-initiated prompting for CRC-S of their primary care physicians increased CRC-S referrals. We wanted to determine if a communication instrument provided to patients initiated a conversation with their primary care physicians about CRC screening, especially *via* colonoscopy. The secondary outcome was to determine whether differences exist in regard to patient-physician communication patterns about screening among residents and faculty in the general internal medicine and family practice clinics. We were also interested in the method of CRC-S given to the patients and the overall acceptance rates for CRC-S among patients.

Statistical analysis

The minimum sample size required to detect a referral frequency difference of 25% after patient initiated prompting was calculated using a confidence level of 95% and confidence interval of 5%. The sample size needed for each group was 52 patients. Differences between groups were analyzed using the unpaired Student's *t*-test for normally distributed data or the Mann-Whitney *U* test for skewed data. The χ^2 test was used for comparisons of categorical variables. Multivariate analysis using stepwise logistic regression was performed to identify independent factors associated with CRC-S referral. All statistical analysis was done using SAS software (v 9.1.3, SAS Institute, Cary, NC). All statistical tests were carried out at an alpha of 0.05.

RESULTS

A total of 274 patients were included from both clinic sites in the present investigation. One hundred forty eight (148) patients were seen in the IMRC and 126 were seen in the FRMC (Figure 1). Among the IRMC patients, 72 (40F:32M) were in the control group and 76 (48F:28M) in the intervention group. In the FRMC patients, 66 (39F:27M) were in the control group and 60 (33F:27M) in the intervention group. No differences were observed in baseline parameters of control or intervention groups from either of the 2 clinics (Table 1). Patient initiated prompting of PCP (intervention) resulted in a significant referral increase for CRC-S in both underinsured and insured patient populations. In the IMRC, 63% in the control group (45/72) got referrals for CRC-S *vs* 92% in

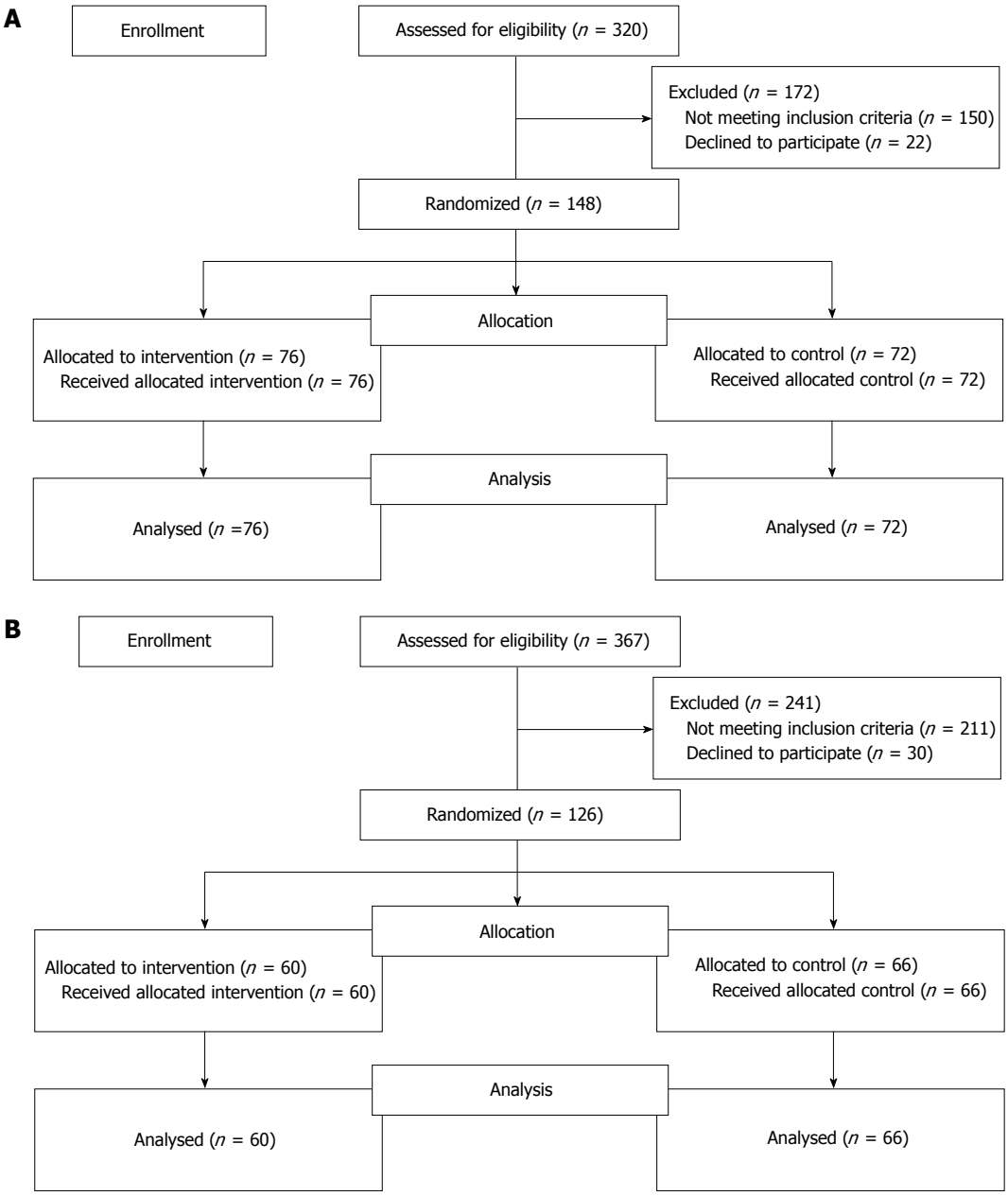

Figure 1 Patient distribution in both clinics between intervention and control groups. A: Internal medicine resident clinic (underinsured); B: Family medicine resident clinic (insured).

the intervention group (70/76, $P \leqslant 0.001$, Figure 2A). In the FMRC, 47/66 (71%) in the control group were referred for CRC-S *vs* 56/60 (98%) in the intervention group ($P \leqslant 0.001$, Figure 2B).

No difference was seen in referral acceptance between the 2 clinics. Among those who got referrals for CRC-S in the IMRC, 31/45 (69%) in the control group *vs* 41/70 (59%) in the intervention group accepted the referrals, (P = NS, Figure 2A). In patients from FMRC who were referred for CRC-S, 36/47 (77%) in the control group *vs* 41/56 (73%) in the intervention group accepted the referral, (P = NS, Figure 2B). In univariate analysis, factors related CRC-S referrals were having insurance (60% *vs* 46%, $P = 0.045$), male gender (38% *vs* 54%, $P = 0.027$), knowledge of CRC recommendations (46% *vs* 26%, $P = 0.0085$) and patients initiated promoting of PCP (intervention) (58% *vs* 18%, $P < 0.0001$). On multivariate logistic regression analysis, male gender (OR = 0.49, 95%CI: 0.26-0.93, $P = 0.03$) and patient initiated promoting the PCP (OR = 6.3, 95%CI: 2.9-13.2, $P < 0.0001$) were identified as independent predictors (Table 2).

All patients referred for CRC-S were offered colonoscopy as the only screening method. Patients were not advised of any other CRC-S method after declining colonoscopy. Overall, 37% of participants in the IMRC and 35% in the FRMC declined CRC-S recommended by the physicians. The primary issue influencing patients' decision to defer CRC-S referral was financial difficulty. Bowel preparation fear, procedure related complications, unsure of colonoscopy benefit, and concern of finding cancer were other, less frequent reasons for not accepting CRC-S referral (Figure 3).

Table 1 Patients characteristics

Characteristics	IMRC		*P* value	FMRC		*P* value
	Control group	Intervention group		Control group	Intervention group	
Number of patients	72	76		66	60	
Median age (range), yr	54 (51-64)	56 (49-70)		55 (48-68)	54 (47-66)	
Sex						
Male	32	28		27	27	
Female	40	48		39	33	
Ethnicity						
Non-hispanic white	35	45		30	32	
African American	26	24		25	19	
Others	9	9		11	9	
Health Insurance						
Yes	12	19		66	60	
No	60	57		0	0	
Education						
< High school graduate	12	25		7	5	
High school graduate	54	41		29	31	
College graduate	6	10		30	24	
Past medical history						
Hypertension	56	60		51	37	
Diabetes mellitus	31	26	NS	25	21	NS
Heart disease	4	7		5	5	
Liver disease	6	6		5	3	
None	12	6		3	9	
Alarm symptoms						
Yes	20	33		28	26	
No	52	43		38	34	
Family history of CRC						
Yes	11	4		12	7	
No	61	72		54	52	
Had a colonoscopy						
Yes	6	11		9	8	
No	66	65		57	52	
Knowledge of CRC recommendations						
Yes	14	26		36	38	
No	58	50		30	22	
Know colonoscopy prevents CRC						
Yes	35	46		42	42	
No	37	30		24	18	

CRC: Colorectal cancer; IMRC: Internal medicine resident clinic; FMRC: Family medicine resident clinic.

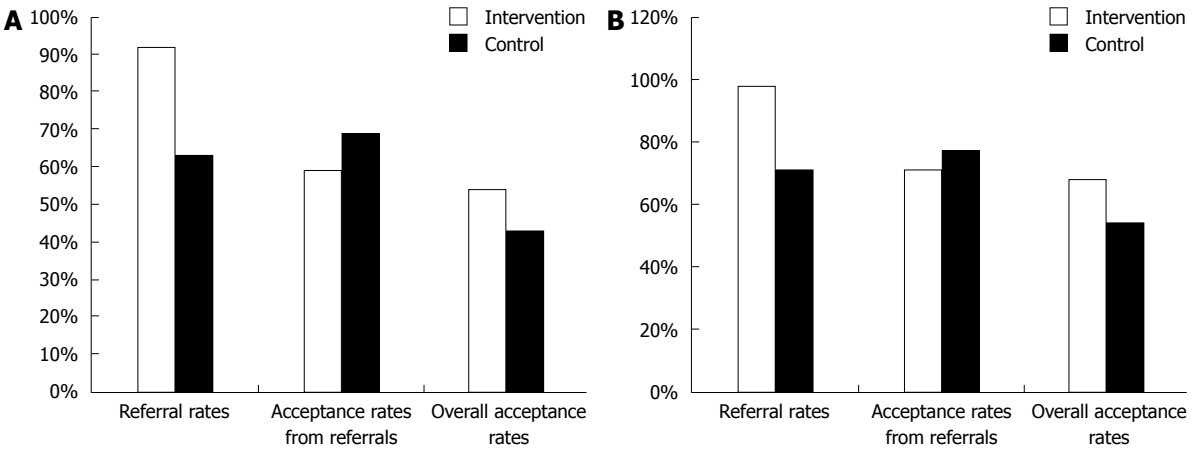

Figure 2 Patterns of referral and acceptance. A: In internal medicine resident clinic (underinsured patients); B: Family medicine resident clinic (Insured patients).

DISCUSSION

Colorectal cancer is the fourth most common cancer diagnosed and second leading cause of cancer related death in the United States[1]. Early stage detection of colorectal cancer has a survival rate of around 80%[1]. Despite the proven efficacy of colorectal cancer screening, only about 50% of eligible patients in the United States are currently

Table 2 Univariate and multivariate analysis of factors impacting colon cancer screening referral *n* (%)

	Offered CRC screening (*n* = 210)	Not offered CRC screening (*n* = 54)	*P* value
Age, mean ± SD	55 ± 4	55 ± 4	0.810
White race	116 (55)	26 (48)	0.350
Male sex	83 (38)	31 (54)	0.027
Higher education	57 (26)	13 (23)	0.590
Insured	131 (60)	26 (46)	0.045
Limiting medical problems	23 (11)	5 (8)	0.680
Symptomatic	91 (42)	16 (28)	0.056
Family history	23 (11)	11 (19)	0.076
Knowledge of CRC recommendations	99 (46)	15 (26)	0.0085
Received pamphlet	126 (58)	10 (18)	< 0.0001
Family medicine providers	102 (47)	24 (42)	0.510

CRC: Colorectal cancer.

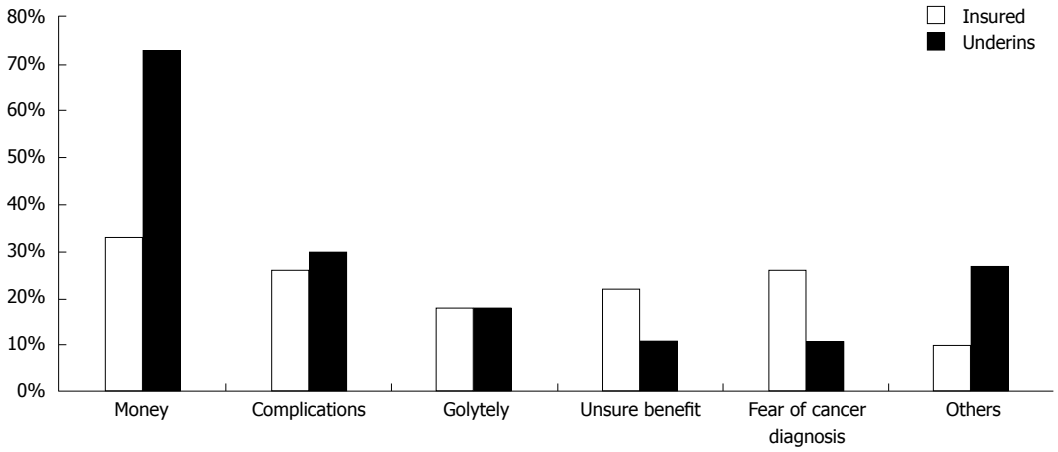

Figure 3 Factors resulting in declining referral between insured and underinsured patients. Underins: Underinsured patients.

being screened[1]. Effective interventions as attempts to increase the referral for CRC-S are lacking. Studies have identified that a lack of communication between physicians and patients was the most common factor resulting in inadequate referrals for CRC-S[2-4]. However, few studies focus on the patient as a factor that contributes to this issue. The primary outcome of our study was to determine if patient initiated prompting of their PCP for CRC-S would increase referrals in both underinsured and insured patients. Increasing patient awareness combined with PCP prompting by patients about CRC-S resulted in increased referral rates.

Among the intervention groups in both clinics, ethnicity did not appear to impact the frequency of patient prompting of physician for CRC-S (data not shown). It is well known that African Americans do not get CRC-S as frequently as non-Hispanic whites[10]. This intervention may help narrow the CRC-S disparity observed, improving long term outcome from this disease.

Multiple barriers to colorectal cancer screening referral by PCPs have been identified in the literature[11-15]. The present study reveals another method where PCPs can be reminded of patient interest in CRC-S and provide appropriate referral for the procedure. This type of intervention using patient prompting of their PCP could decrease the burden on the PCP to remember appropriate CRC-S recommendations, resulting in an increased screening rate overall.

Referral rates after intervention were found to be increased in both clinic populations but acceptance rates after referral were less in both intervention groups, unexpectedly. This resulted in lower overall acceptance rates for both clinics and was not significantly different between intervention or control groups. Multiple factors have been identified which contribute to a reduced acceptance rate for CRC-S[16]. In our study, multiple issues were evident. First, college education was more prevalent in patients with medical insurance coverage and more of these individuals were aware of current CRC-S literature than underinsured patients. However, this did not impact whether CRC screening was offered. Secondly, we observed a higher acceptance rate, in insured patients, for CRC-S offered by their primary physicians compared to the underinsured which has been reported by previous investigators[17-19]. Finally, acceptance rate for CRC-S was increased in patients with alarm symptoms compared to asymptomatic patients in both control and intervention groups. The most common limiting factor influenced patient's decision to refuse CRC screening was financial affordability in both underinsured (72%) and insured

populations (36%) even though significantly lower in the insured population. Procedure complications, bowel preparation concerns, colonoscopy benefit uncertainty, and fear of finding cancer were other less common reasons for not accepting referrals.

A limitation to the present study is not using other screening methods available if colonoscopy is declined. As colonoscopy was considered the test of choice and other methods, if positive, result in colonoscopy referral, use of alternative screening tools appeared redundant to the investigators. However, some individuals may prefer colonoscopy only following a positive result from another screening tool and should be considered in larger scale investigations.

CRC-S referrals significantly increased with patient initiated prompting of physicians for such screening. Larger investigations, using this method, directed towards increasing acceptance of CRC-S are warranted.

COMMENTS

Background

Despite the available evidence suggesting the effectiveness of colorectal screening (CRC-S), almost half of the United States population over 50 years has not been tested. According to the National Cancer Institute, in 2009 the estimated new cases of colon cancer and rectal cancer in United States were 106100 and 40870 respectively.

Research frontiers

Effective interventions to increase patient referrals for CRC-S are lacking. Studies have identified that a lack of communication between physicians and patients was the most common factor resulting in inadequate referrals for CRC-S.

Innovations and breakthroughs

As colonoscopy was considered the test of choice and other methods, if positive, result in colonoscopy referral, use of alternative screening tools appeared redundant to the investigators.

Peer review

This is a well constructed study, of high clinical significance. It seems that it is sufficiently powered to detect pre-specified 25% difference in referral frequency, but in my opinion this sample size is not sufficiently enough to portray independent predictors resulting in declining referral between insured and underinsured patients.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flex-

ible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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