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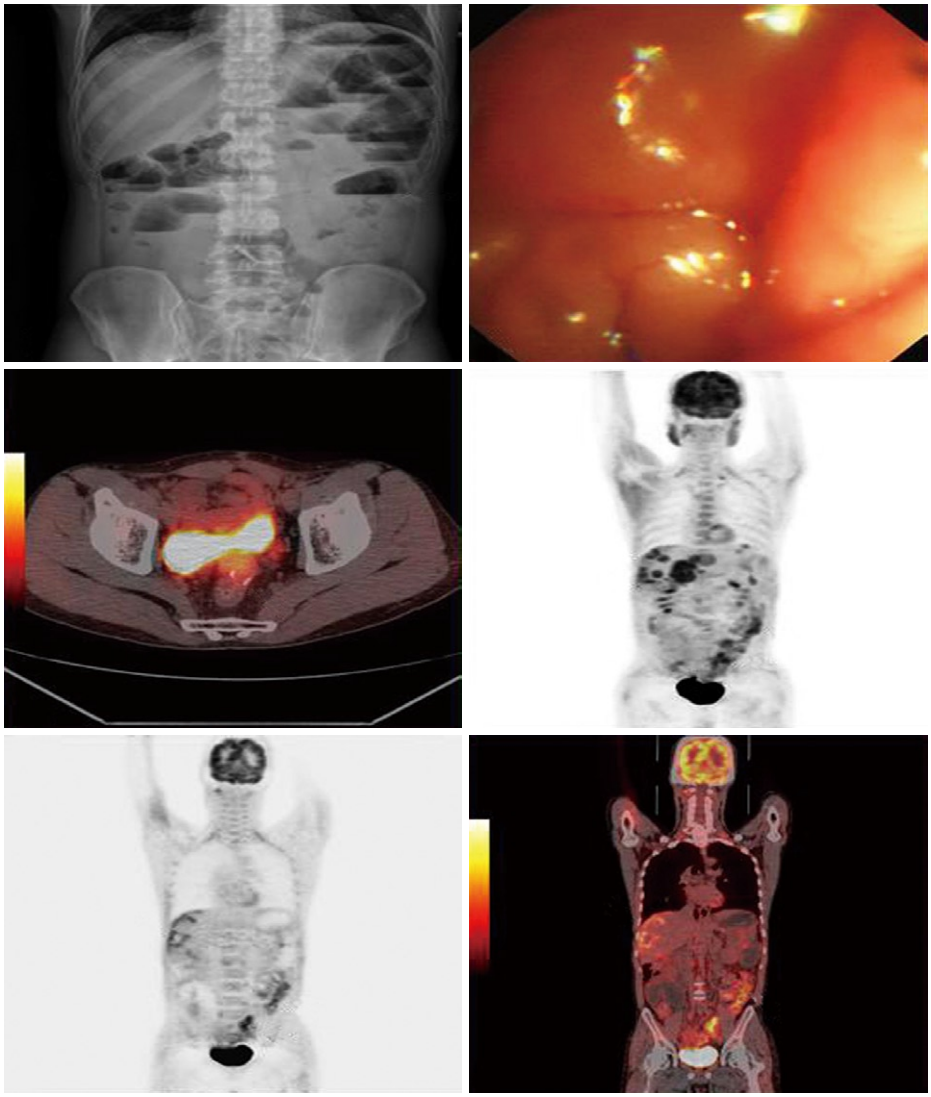

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Fig 2C | Fig 2D
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2009-2013

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EDITORIAL

What is the purpose of launching *World Journal of Gastrointestinal Oncology*?

Lian-Sheng Ma

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Abstract

The first issue of *World Journal of Gastrointestinal Oncology* (*WJGO*), whose preparatory work was initiated on September 27, 2008, will be published on October 15, 2009. The *WJGO* Editorial Board has now been established and consists of 210 distinguished experts from 32 countries. Our purpose of launching *WJGO* is to publish peer-reviewed, high-quality articles *via* an open-access online publishing model, thereby acting as a platform for communication between peers and the wider public, and maximizing the benefits to editorial board members, authors and readers.

Key words: Maximization of personal benefits; Editorial board members; Authors; Readers; Employees; *World Journal of Gastrointestinal Oncology*

Ma LS. What is the purpose of launching *World Journal of Gastrointestinal Oncology*? *World J Gastrointest Oncol* 2009; 1(1): 1-2 Available from: URL: http://www.wjgnet.com/1948-5204/full/v1/i1/1.htm DOI: http://dx.doi.org/10.4251/wjgo.v1.i1.1

INTRODUCTION

I am very pleased to announce that the first issue of *World Journal of Gastrointestinal Oncology* (*World J Gastrointest Oncol*, *WJGO*, ISSN 1948-5204, DOI: 10.4251) will be published on October 15, 2009. Originally, the journal was titled *Gastrointestinal Cancer Review Letters* when preparatory work was initiated on September 27, 2008. The *WJGO* Editorial Board has now been established and consists of 210 distinguished experts from 32 countries.

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. To realize these desired attributes of a journal and create a well-recognized journal, the following four types of personal benefits should be maximized.

MAXIMIZATION OF PERSONAL BENEFITS

The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others.

Maximization of the benefits of editorial board members

The primary task of editorial board members is to give a peer review of an unpublished scientific article *via* online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board

members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution.

Maximization of the benefits of authors

Since *WJGO* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJGO* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading.

Maximization of the benefits of readers

Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion[1].

Maximization of the benefits of employees

It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal[2,3]. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

CONTENTS OF PEER REVIEW

In order to guarantee the quality of articles published in the journal, *WJGO* usually invites three experts to comment on the submitted papers. The contents of peer review include: (1) whether the contents of the manuscript are of great importance and novelty; (2) whether the experiment is complete and described clearly; (3) whether the discussion and conclusion are justified; (4) whether the citations of references are necessary and reasonable; and (5) whether the presentation and use of tables and figures are correct and complete.

SCOPE

The major task of *WJGO* is to report rapidly the most recent advances in basic and clinical research on gastrointestinal oncology. The topics of *WJGO* cover the carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. This cover epidemiology, etiology, immunology, molecular oncology, cytology, pathology, genetics, genomics, proteomics, pharmacology, pharmacokinetics, nutrition, diagnosis and therapeutics. This journal will also provide extensive and timely review articles on oncology.

COLUMNS

The columns in *WJGO* will include: (1) Editorial: to introduce and comment on major advances in rapidly developing areas and their importance; (2) Frontier: to review recent developments, comment on current research status in important fields, and propose directions for future research; (3) Topic Highlight: this column consists of three formats, including: (a) 10 invited review articles on a hot topic; (b) a commentary on common issues associated with this hot topic; and (c) a commentary on the 10 individual articles; (4) Observation: to update the development of old and new questions, highlight unsolved problems, and provide strategies for their resolution; (5) Guidelines for Basic Research: to provide guidelines for basic research; (6) Guidelines for Clinical Practice: to provide guidelines for clinical diagnosis and treatment; (7) Review: to review systemically the most representative progress and unsolved problems, comment on current research status, and make suggestions for future work; (8) Original Article: to report original and innovative findings; (9) Brief Article: to report briefly on novel and innovative findings; (10) Case Report: to report a rare or typical case; (11) Letters to the Editor: to discuss and reply to contributions published in *WJGO*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: to introduce and comment on quality monographs; and (13) Guidelines: to introduce consensuses and guidelines reached by international and national academic authorities on basic research and clinical practice.

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EDITORIAL

Telomere function in colorectal cancer

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Author contributions: Frías C, Morán A, de Juan C, Ortega P and Fernández-Marcelo T performed the molecular analyses; Sánchez-Pernaute A and Torres AJ assessed the clinical correlations; Díaz-Rubio E was involved in the work; Iniesta P directed and coordinated this work; Iniesta P, Benito M, Frías C and Morán A wrote this manuscript.
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Abstract

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the western world. Tumour cells acquire the hallmarks of cancer during the carcinogenic selection process. Cell immortality is one of the principal features acquired during this process which involves the stabilization of telomere length. It is achieved mainly, by telomerase activation. Thus, the discovery of telomeres and telomerase allowed an understanding of the mechanisms by which cells can become immortalized. Different studies have shown that tumour cells have shorter telomeres than nontumour cells and have detected telomerase activity in the majority of tumours. Survival studies have determined that telomere maintenance and telomerase activity are associated with poor prognosis. Taking into account all the results achieved by different groups, quantification and evaluation of telomerase activity and measurement of telomere length may be useful methods for additional biologic and prognostic staging of colorectal carcinoma.

Key words: Colorectal cancer; Telomeres; Immortality

Peer reviewer: Reigetsu Yoshikawa, MD, PhD, Assistant Professor, Department of Genetics, and Department of Surgery, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

Frías C, Morán A, de Juan C, Ortega P, Fernández-Marcelo T, Sánchez-Pernaute A, Torres AJ, Díaz-Rubio E, Benito M, Iniesta P. Telomere function in colorectal cancer. *World J Gastrointest Oncol* 2009; 1(1): 3-11 Available from: URL: http://www.wjgnet.com/1948-5204/full/v1/i1/3.htm DOI: http://dx.doi.org/10.4251/wjgo.v1.i1.3

INTRODUCTION

Colorectal cancer (CRC) is the third most common form of cancer and the second leading cause of cancer-related death in the western world. More than 940 000 cases occur annually worldwide and nearly 500 000 die from it each year. It represents 9.5% of all cancers diagnosed and there is a high incidence in USA, Australia, Japan and Europe. Sporadic colorectal cancers account for 70%-80% of cases whereas familiar syndromes constitute up to 25% and are usually diagnosed at early ages. A major cause for sporadic CRC is a diet rich in fat, refined carbohydrates and animal protein, combined with low physical activity. Therapy is usually through surgery, which in many cases is followed by chemotherapy. Overall five-year survival is around 50% in Europe. It has been improving during the last decade and this tendency is expected to continue[1,2].

Colorectal cancer is a disease originating from the epithelial cells lining the gastrointestinal tract. Hereditary or somatic mutations in specific DNA sequences, among which are included DNA replication or DNA repair genes, and also the *APC*, *K-Ras*, *NOD2* and *p53* genes lead to unrestricted cell division[3,4].

Carcinogenesis is a multistep, multifocal process characterized by the accumulation of genetic and molecular abnormalities. It is well known that normal human somatic cells have a finite limit of cultivation when grown in vitro unless immortalization protocols are carried out. Current knowledge suggests that the progression of cancer from a premalignant to a malignant state is, consistent with a mechanistic model, based on of the principle of natural selection. Tumour cells acquire the hallmarks of cancer during this carcinogenic selection process[5]. Cell immortality is one of the principal features acquired during this process which involves the stabilization of telomere length. It is achieved, mainly, by telomerase activation. Thus, the discovery of telomeres and telomerase allowed an understanding of the mechanisms by which cells can become immortalized[6].

Telomeres are essential for chromosomal stability and integrity, protecting the ends of chromosomes from degradation and preventing chromosomal end fusions and recombination[7]. Indeed, loss of telomere function can be a major mechanism for the generation of chromosomal abnormalities[8,9]. Telomeres are the end cap on chromosomes and consist of repetitions of six nucleotides. In humans, the sequence is 5'-(TTAGGG)$_n$-3' and they vary in length from 5 to 15 Kb[10-12]. Telomeres end in an essential 3' single-stranded overhang of 50 to 400 nt[13-15]. Electron microscopy studies suggest this overhang can loop back and integrate into the duplex repeat tract, forming a "t-loop" [16]. Due to replication deficiencies (end replication problems) and telomere end processing, telomeres shorten progressively with replication in normal somatic cells and, eventually, trigger senescence. Cells that lose the ability to senesce because of mutations in p53 protein continue dividing, till they enter "crisis", where extensive telomere shortening results in chromosomal fusion and cell death. This telomere length-dependent growth inhibition which prevents critically short telomeres and, thereby, potentially unprotected chromosomes, is thought to be a barrier for unlimited cellular proliferation[17-22].

A group of proteins also play important roles in the regulation of telomere length maintenance and in the formation of a protective end-cap that prevents chromosome fusion. The mammalian telomeric core complex serves to form and protect the telomere, and has been termed shelterin. This complex includes proteins that bind directly to the telomeric DNA (TRF1, TRF2 and POT1) and telomere-associated proteins that are recruited to telomeres by the former (TIN2, TPP1 and Rap1)[23,24]. Other proteins, many of which are more commonly associated with DNA repair, are also found at telomeric ends. Examples include DNA-PK, the MRN complex, PARP1/2, Tankyrase 1/2, ATM, ERCC1/XPF, RAD51D, WRN and BLM[25].

It has been postulated that dysfunctional telomeres could play a causal role in carcinogenesis by instigating chromosomal instability, thus promoting neoplastic transformation[26-30]. Results from telomerase-knockout mouse models in which animals possessing critically short telomeres exhibit an increased cancer incidence support this concept[31,32]. In particular, Artandi *et al*[33] demonstrated that crossing telomerase- knockout mice with *p53* +/- mice resulted in a shift in the spectrum of tumours normally seen in the *p53*-defective background (primarily lymphomas and sarcomas) to one dominated by carcinomas displaying the types of karyotypic aberrations (e.g. nonreciprocal translocations) often observed in human epithelial cancers.

Human telomerase was discovered for the first time in cancer cells (HeLa) in 1989. The core enzyme consists of two subunits: the RNA component (hTR) which serves as a template for telomere synthesis and a catalytic protein, the telomerase reverse transcriptase (hTERT)[34-36]. Together, the telomerase ribonucleoprotein complex is responsible for adding telomere repeats to the very ends of chromosomes and thus compensates for replication- or damage-dependent loss of telomere sequences[37]. Over expression of telomerase has been found in more than 90% of human cancers, leading to the hypothesis that telomerase plays an important role in the pathogenesis of cancer[38-40]. Given the importance of telomerase to tumourigenesis, multiple studies have assessed the use of pharmacological, immunological and targeting technologies to diminish or abolish the expression of telomerase hTR and hTERT as a novel therapeutic strategy for cancer[40].

Some tumours, around 7%-10%, do not express telomerase and they maintain their telomeres through a mechanism termed alternative lengthening of telomeres or ALT[41]. ALT-positive cells are characterized by very long telomeres (> 40 Kb) as well as an extremely large variation in telomere length within the same nucleus. Another hallmark of the ALT mechanism is the presence of ALT-associated promyelocytic leukaemia protein (PML) nuclear bodies, the APBs, subnuclear structures containing PML protein, telomeric DNA, telomere-binding proteins and several proteins involved in DNA synthesis and recombination[42].

Telomere lengths are the result of the balance of proliferate telomere losses and *de novo* telomere synthesis. They serve as an indicator of the ability of each tumour to compensate for replicative telomere losses. When examined by Southern blotting analysis (Figure 1), the telomeres of invasive human cancers often appear shorter than their normal tissue counterparts[28]. The combined observations of short telomeres, plus the frequent activation of telomerase in human epithelial cancers, suggest that the majority of tumours undergo critical telomere shortening at some point during their development. This could simply be a consequence of the end-replication problem combined with extensive cell turnover occurring during tumour expansion. On

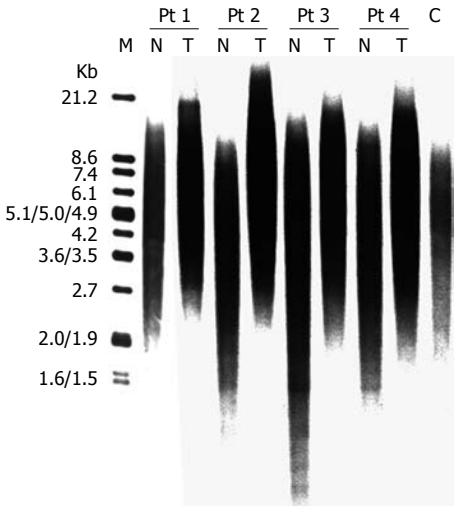

Figure 1 Representative X-ray film for telomere length analysis by Southern blotting. Pt1-Pt4: Patients with colorectal carcinoma; T: Tumour tissues; N: Their paired normal samples; C: Positive control; M: DNA molecular weight marker; kb: Kilobase pairs.

the other hand, if telomere shortening occurs early, it could be playing an important role during the initiation stage of tumourigenesis. Thus, the timing of the occurrence of telomere shortening during human cancer development is a critical question[5].

TELOMERES AS MOLECULAR PROGNOSTIC FACTORS IN COLORECTAL CANCER

Two studies carried out in 57 and 91 patients showed that median telomere lengths, measured by Southern blotting, in cancer tissue were significantly shorter than matched adjacent mucosa ($P < 0.001$ and $P = 0.020$, respectively)[43,44]. A significant positive correlation was observed between nontumour telomere length and tumour telomere length by Garcia-Aranda *et al*[44] and Nakamura *et al*[45]. Patient-by-patient comparison of matched tissue samples showed that 86% of samples (49 tumours) had telomere shortening[43]. Similar results were obtained by Engelhardt *et al*[46] and Nakamura *et al*[45] who found telomere shortening in 90% and 77% of colon tumours, respectively. Tatsumoto *et al*[47] arbitrarily defined TRFs as shortened when TRF length of tumour tissues was shorter than 80%. Among the primary cancers, Garcia-Aranda *et al*[44] and Tatsumoto *et al*[47] detected the shortened TRF lengths in 38% and 39.6% of tumours, respectively. The mean ratio of telomere lengths in cancer tissue to corresponding non cancerous mucosa was very similar in two different studies: 0.84 and 0.87[43,44].

The clinical significance of a correlation between telomere length in CRC tissue and stage is controversial. Some reports reveal a significant association between telomere length and stage in cancer tissue[43,45]. Thus, according to Gertler *et al*[43] telomeres in early-stage tumours (UICC stage Ⅰ) were significantly shorter than telomeres of advanced tumours (UICC stages Ⅱ through Ⅳ). The telomere length ratio of cancer to non-cancerous tissue increased within higher stage groups, approaching statistical significance ($P < 0.060$)[43]. Engelhardt *et al*[46] also reported on significantly longer telomeres in late-stage Dukes' C and D tumours compared with early stage Dukes' A and B tumours. Garcia-Aranda *et al*[44] failed to find this association. However, they defined a correlation between tumour telomere length and tumour location ($P = 0.005$) and tumour differentiation ($P = 0.046$). With this regard, tumours of the right colon displayed significantly shorter telomeres compared with tumours located in other sites. When considering tumour status (telomere shortening, maintainance or elongation), borderline-significant differences were observed for tumour differentiation ($P = 0.098$). Well differentiated and moderately differentiated tumours demonstrated telomere maintenance, whereas telomere dysfunction was detected in the majority of poorly differentiated tumours. Tatsumoto *et al*[47] assessed there was no significant relationship between altered TRF length and Dukes' classification nor other clinic-pathological parameters (tumour site, tumour size, lymph node metastasis, or histology), although mean telomere length in advanced stages tumours was slightly longer than that of early stage tumours (Table 1). Other available studies did not associate telomere length with tumour stage or depth of tumour invasion in patients with colorectal carcinoma[48-50].

O'Sullivan *et al*[51] have shown that telomere dysfunction is an early event in neoplastic progression in ulcerative colitis, and is related to chromosomal instability and anaphase bridge formation. This may facilitate the molecular evolution of tumourigenesis in cells by accelerating chromosomal instability. Recently, Raynaud *et al*[52] showed that telomere length depended on stage in CRC. They determined by FISH that the decrease in staining with the transition from normal tissue to LGD and with that from LGD to HGD was statistically significant ($P < 0.0001$ and $P = 0.012$, respectively). During later stages of the carcinogenic process, staining increased with invasiveness, reaching the levels observed in normal cells in invasive carcinoma. This increase in staining associated with the transition from HGD to IC was also statistically significant ($P = 0.006$). This attrition peaks in HGD, and it is only when the full invasive potential of the tumour has been reached that telomere length returns to levels close to those observed in normal tissue. Raynaud *et al*[52] hypothesized that this telomere attrition may have a protective effect against cancer because critically short telomeres are known to induce replicative senescence. Consequently, only cells that find a way to maintain their telomeres, thus allowing unlimited cell division and some degree of genomic stabilization, will be able to pass through this bottleneck and progress to give rise to an invasive tumour[5]. Meeker *et al*[5] support a model whereby telomere dysfunction induces chromosomal instability as an early initiating event in many, perhaps most, human epithelial cancers. Plentz *et al*[53] agree with this model. They showed for the first time that telomere

Table 1 Summary of the relationship between telomere length and clinic-pathological parameters in CRC described by different authors

Authors	No. of CRC patients	Telomeres and clinic-pathological parameters
Engelhardt *et al*[46], 1997	100	Telomeres in early stage Dukes' A and B tumours were significantly shorter than telomeres in late-stage Dukes' C and D tumours
Gertler *et al*[43], 2004	57	Telomeres of UICC stage I tumours were significantly shorter than telomeres of UICC stages Ⅱ-Ⅳ tumours. Telomere length ratio of cancer to no cancer tissue was shown to be an independent prognostic parameter for overall survival. Telomere length ratio > 0.90 implied a 3.3 times higher RR compared with patients who had telomere shortening
Garcia-Aranda *et al*[44], 2006	91	Telomeres of tumours of the right colon displayed significantly shorter telomeres compared with tumours located in other sites. A significantly poor clinical outcome in the group of patients who had tumours with longer telomeres was observed both in overall survival and disease-free survival. This parameter was found to be a significant prognostic factor independent of tumour stage. RR was 6.48 in patients who had tumours with longer telomeres

CRC: Colorectal cancer.

shortening characterizes the adenoma-carcinoma transition and that telomere shortening at this transition specifically affects epithelial cells, the cell type of origin of colorectal cancer. These authors suggest that carcinomas arise from chromosomally unstable epithelial cells with critical short telomeres which have lost DNA damage responses. Subsequently or simultaneously, a variety of cofactors are necessary for cancer progression, including activation of telomerase to stabilize telomeres and alleviate chromosomal instability to a level allowing further cell divisions and cell survival. It should also be considered that several proteins have been implicated in the formation of a protective higher-order capping structure at the telomeres, and experimental changes in the level of expression or function of several of these proteins have been shown to affect telomere length, both positively and negatively[45,54,55]. Taken together, telomeres may rapidly shorten as a result of inefficiently repaired DNA damage caused by oxidative stress[56].

Different results were presented by Engelhardt *et al*[46] who measured telomere length in premalignant lesions, CRCs and adjacent normal tissues by Southern blotting. Telomeres in colon cancer were considerably shorter than those in adjacent normal tissues ($P = 0.002$), whereas polyps and colitis had telomere lengths comparable to those of the normal adjacent tissues ($P = 0.312$ and $P = 0.830$, respectively).

Takagi *et al*[57] studied the relationship between microsatellite instability and telomere shortening in colorectal cancer. They assessed that microsatellite instability correlated significantly with frequency of telomere shortening ($P = 0.018$) and concluded that the relationship identified between microsatellite instability and telomere shortening might suggest some association between the DNA mismatch repair system and the telomere maintenance mechanism in colorectal cancers.

Patients with tumours that maintain telomere length showed an increased hazard rate in CRC. Gertler *et al*[43] described a 5-year survival of 25.6% ± 13.8% in patients with a TRF T/TRF N greater than 0.9, whereas those with a ratio ⩽ 0.9, showed a 5-year overall survival rate of 78.2% ± 6.9% ($P < 0.002$). In multivariate analysis (Cox regression), the telomere length ratio of cancer to non cancerous tissue was shown to be an independent prognostic parameter for overall survival ($P < 0.03$). The relative risk (RR) of death for patients with a telomere length ratio > 0.90 was 3.3 times higher compared with patients who had telomere shortening (95% CI: 1.2 to 9.0). The only other independent prognostic factor for overall survival was lymphatic vessel invasion, with a relative risk of 4.1 (95% CI: 1.5 to 11.6; $P < 0.01$).

Garcia-Aranda *et al*[44] considered two groups to analyze the impact of telomere length in patient prognosis. Group 1 included patients in the first and second quartiles and Group 2 included patients in the third and fourth quartiles. A significantly poor clinical outcome in the group of patients who had tumours with longer telomeres was observed both in overall survival and disease-free survival ($P = 0.020$ and $P = 0.060$, respectively). The differences found between these analyses derived from the fact that three patients who had recurrent tumours did not die during follow-up; on the other hand, one patient without tumour recurrence died because of further complications. Using the multivariate Cox proportional hazards model, this parameter was found to be a significant prognostic factor independent of tumour stage ($P = 0.040$). The relative risk was 6.48 in patients included in Group 2 (Table 1).

TELOMERASE AS A COLORECTAL CANCER MARKER

The most frequently used method to evaluate telomerase activity is the Telomere Repeat Amplification Protocol (TRAP assay), originally described by Kim *et al*[38] (Figure 2). Previous studies demonstrated increased telomerase activity in colorectal cancer tissue and even suggested a prognostic value for patients with colorectal carcinoma[45].

Chadeneau *et al*[58] described an association between telomerase activity and acquisition of malignancy in human colorectal cancer. Engelhardt *et al*[46] and Boldrini *et al*[59] found that dysplastic polyps and adenocarcinoma samples were telomerase positive, with higher levels in cancer tissues compared to dysplastic lesions. Engelhardt *et al*[46] postulated that some of the early-stage adenomas

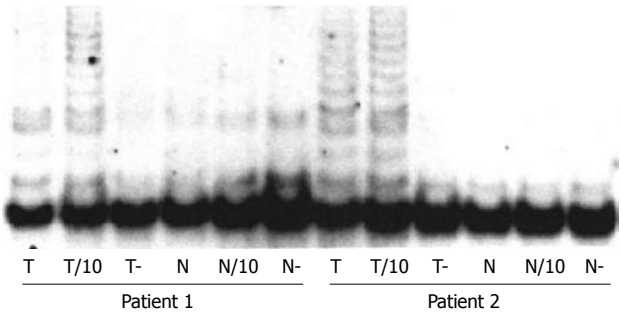

Figure 2 Telomerase activity in tissue extracts from two patients affected by colorectal cancer. In this representative experiment, telomerase activity was investigated in tumour (T) and non tumour (N) samples using the telomerase repeat amplification protocol (TRAP). As it is indicated in the figure, to avoid the effect of Taq DNA polymerase inhibitors, different extract dilutions were investigated.

may be telomerase negative, because they have not acquired all of the genetic abnormalities compared to the later-stage adenomas. Therefore, multistep genetic alterations in the preneoplastic development of adenomas may be involved in the selection of cell immortality and may lead to expression of telomerase activity in the more advanced adenomas. Boldrini *et al*[59] determined that high telomerase activity was associated with late-staged cancers and metastasis, what provided arguments supporting the role of telomerase not only in the development but also in the progression of colorectal carcinoma.

In studies carried out in a total of 17, 30, 41, 50, 91, 97, 100, 103 and 108 patients diagnosed with colon adenocarcinoma, positive telomerase activity was detected in 82.4% (14 tumours), 100% (30 tumours), 83% (34 tumours), 100% (50 tumours), 81.3% (74 tumours), 92.8% (90 tumours), 96% (96 tumours), 90% (93 tumours) and 81.5% (88 tumours) of the colon carcinoma samples, respectively[44,45,47,60-65].

Telomerase activity is related to the Dukes' stage: patients at stage A and B showed a lower percentage of positivity than the ones at stages C or D[45,47,61,62,64,66]. Tumours localized in colon showed a higher percentage of positivity than tumours localized in the rectum[61,64]. Maláska *et al*[65] and Okayasu *et al*[67] reported a correlation between positive telomerase activity and lymph node metastasis. Moreover, Maláska *et al*[65] found a tendency towards higher telomerase activity in patients with distant metastases, although this lacked statistical significance. Shoji *et al*[60] found that telomerase index (TI = log telomerase activity of cancer tissue - log telomerase activity of normal mucosa) increased significantly with depth of invasion ($P = 0.013$). Additionally, there was a significant difference in TI between tumours with and without venous invasion ($P < 0.001$): TI was higher in telomerase positive tumours. Garcia-Aranda *et al*[44] described tumour recurrence as a borderline-significant parameter, because all tumours which were positive for recurrence during follow-up showed telomerase activity ($P = 0.068$). These authors established an association between telomerase positive tumours and age and gender: higher rates of telomerase activity were detected in the group of patients older than 69 years-old ($P = 0.003$) and in females ($P = 0.057$) (Table 2). However, other authors disagree as they found no significant association between telomerase activity in tumour or normal mucosa related to clinical variables (gender and age) or to the anatomopathologic characteristics (histopathological grade, histology type, tumour localization, depth invasion (T), lymph node invasion (N), distance metastasis (M), and stage TNM classification)[63,68].

Kawanishi-Tabata *et al*[69] analysed 122 surgical specimens of human stage Ⅱ colorectal carcinoma. Telomerase activity was detected in 98 tumours (80%). The colon was the primary site of the tumour in 52 cases, whereas the rectum was the primary tumour site in 70 cases. Among the 52 colon tumour specimens evaluated, 47 (90%) were telomerase positive, whereas only 51 of the 70 rectal tumour specimens (73%) were positive for telomerase activity ($P = 0.020$). These results showed concordance with the ones presented by Lukman *et al*[61] and Sanz-Casla *et al*[64]: more patients with colon than with rectal carcinomas were telomerase positive in the series they analysed. Kawanishi-Tabata *et al*[69] also found that telomerase positive tumours presented more frequently in females ($P = 0.100$), as Garcia-Aranda *et al*[44], and were larger ($P = 0.040$) (Table 2).

Neither Garcia-Aranda *et al*[44] nor Tatsumoto *et al*[47] reported significant differences between TRF lengths in telomerase positive tumours versus telomerase negative tumours in CRC.

Tatsumoto *et al*[47] were the first to associate telomerase activity levels and patient prognosis. Kaplan-Meier overall survival curves of 100 patients showed that 5-year survival rate in the patients with high telomerase activity was 43%, whereas that in all remaining patients was 81%. The prognosis of patients with high telomerase activity was significantly worse than those for other patients ($P < 0.010$). Disease-free survival curves of 87 patients after curative surgery, showed significant difference between the tumour-free survival rates with and without high telomerase activity ($P < 0.010$). Among these patients who underwent curative surgery, 13 (38%) of 34 tumours with high telomerase activity recurred, whereas only 7 (13%) of 52 other tumours did ($P = 0.016$). Thus, curative tumours with high telomerase activity had significantly higher recurrence rates than other tumours. Considering that telomerase activity levels did not significantly correlate with stage of disease or Dukes' classification in the present study, the up-regulation of telomerase activity was considered an independent prognosis-associated factor in patients with colorectal cancer (Table 2).

Sanz-Casla *et al*[64] performed multivariate analysis to study the impact of telomerase activity in prognosis in 103 patients undergoing surgery for colorectal cancer. These data revealed that by adjusting for tumour stage, telomerase activity could be used to predict the risk of death or recurrence ($P < 0.001$) (Table 2).

Garcia-Aranda *et al*[44] identified telomerase activity as a marker of a trend toward a poor prognosis. Although no significant differences were found between patients

Table 2 Summary of the relationship between telomerase activity and clinic-pathological parameters in CRC described by different authors

Authors	No. of CRC patients	Telomerase and clinic-pathological parameters
Engelhardt *et al*[46], 1997	100	Lower rates of telomerase activity were detected in tumours at early stages
Okayasu *et al*[67], 1998	37[1]	There is a correlation between positive telomerase activity and lymph node metastasis
Lukman *et al*[61], 2000	17	Lower rates of telomerase activity were detected in tumours at early stages. Telomerase activity detection is more frequent in colon than in rectal tumours
Shoji *et al*[60], 2000	30	TI increased significantly with depth of invasion. TI was higher in telomerase positive tumours with venous invasion
Tatsumoto *et al*[47], 2000	100	Lower rates of telomerase activity were detected in tumours at early stages. The overall survival and the disease-free survival of patients showing high telomerase activity was significantly worse than those for other patients. Telomerase activity was an independent prognosis-associated factor
Ghori *et al*[66], 2002	30/20[2]	Lower rates of telomerase activity were detected in tumours at early stages
Kawanishi-Tabata *et al*[69], 2002	122[1]	Telomerase activity detection is more frequent in colon than in rectal tumours at stage Ⅱ. Telomerase positive tumours at stage II were larger than telomerase negative tumours. Disease-free survival for patients with telomerase negative tumours was shorter than for patients with telomerase positive tumours
Maláska *et al*[65], 2004	41	There is a correlation between positive telomerase activity and lymph node metastasis
Sanz-Casla *et al*[64], 2005	103	Lower rates of telomerase activity were detected in tumours at early stages. Telomerase activity detection is more frequent in colon than in rectal tumours. Telomerase activity could be used to predict the risk of death or recurrence
Garcia-Aranda *et al*[44], 2006	91	Higher rates of telomerase activity were detected in patients older than 69 years-old. Patients who had tumours with telomerase activity and high telomere length ratios had a significantly shorter disease-free survival compared with patients whose tumours showed lower telomere length ratios
Bautista *et al*[72], 2007	108	Patients with low TI rectal tumours showed a higher recurrence-free survival and overall survival probability. TI was an independent prognostic factor for predicting the recurrence in the first two years after surgery and for survival in rectal cancer patients
Vidaurreta *et al*[62], 2007	97	Lower rates of telomerase activity were detected in tumours at early stages. The overall survival of patients with telomerase activity was significantly worse than those for other patients

[1]Number of tumour samples; [2]30 tumour samples and 20 adjacent normal mucosa samples.

with telomerase positive tumours and patients with telomerase negative tumours, no recurrences were detected in the latter, during follow-up (P = 0.110). These differences translated into differences in overall survival. Therefore, this study revealed that no deaths were detected in the group of patients with telomerase negative tumours (P = 0.110). In addition, patients who had tumours with telomerase activity and high telomere length ratios had a significantly shorter disease-free survival compared with patients whose tumours showed lower telomere length ratios (P = 0.030) (Table 2).

Vidaurreta *et al*[62] determined overall survival and found that none of the patients with negative telomerase died during the follow-up period (P = 0.040). It should be considered that 53.6% of the population was subjected to adjuvant chemotherapy based on 5-fluorouracil (5-FU). These authors found no significant differences between the group of treated patients and the group of non-treated patients, with respect to the adjuvant chemotherapy treatment, when it was stratified by stages. These results are supported by previous ones which assessed that patients who underwent chemotherapy with 5-FU and their tumours had low telomerase activity, showed a tendency to chemo sensitivity (Table 2).

Recently, Ohnishi *et al*[63] evaluated telomerase activity from peripheral blood samples in 120 CRC patients who underwent curative surgical treatment. In univariate analysis, they found recurrence was significantly correlated with positive telomerase activity in the mesenteric vein (P = 0.002), positive telomerase activity in the peripheral vein (P = 0.003), histological type except well differentiated adenocarcinoma (P = 0.001), lymphatic infiltration (P = 0.044) and lymph node metastasis at surgery (P < 0.0001). In multivariate analysis, positive telomerase activity in peripheral vein was significantly associated with the existence of recurrence (HR: 3.13; P = 0.037). Ohnishi *et al*[63] assessed that measuring telomerase activity in peripheral blood samples seemed to be effective in predicting future recurrence to a degree greater than macroscopically examined tumour depth or other clinicopathological parameters.

Contrary to the results presented in this review, there is one study in which neither telomerase nor telomere length were predictive factors for overall survival[45]. However, patients with a very short survival (< 10 mo), all of whom had Dukes C (n = I) and Dukes D (n = 4) tumour stages, had high telomerase activity and short telomeres, whereas patients with extended survival (> 45 mo) had significantly lower telomerase activity and longer telomeres.

On the other hand, telomerase activity emerged as the only factor impacting disease-free survival (P = 0.050) in a series of patients with stage Ⅱ tumours[69] (Table 2). The authors of this study determined that the prognosis for patients with telomerase negative tumours was worse than that for patients with telomerase positive tumours. No association was found between telomerase activity and overall survival. They discussed that the poor prognosis observed in telomerase positive patients by other groups may, in some cases, represent an advanced stage of disease. In addition, tumours lacking both telomerase

and p53 could have a worse prognosis than telomerase positive tumours, as telomere shortening leads to mutations, chromosome rearrangements and translocations, which promote cellular transformations. Moreover, defects in mismatch repair genes, which have been reported in hereditary nonpolyposis colorectal carcinoma and spontaneous tumours, may facilitate cell proliferation and survival in the absence of telomerase due to activation of a recombination-dependent pathway for telomere maintenance and the accumulation of tumour-promoting mutations[69-71].

Bautista *et al*[72] determined TI to classify tumours in 2 groups: tumours with low telomerase index and tumours with high telomerase index. In 54 patients with colon cancer, they did not find a significant association between either recurrence-free survival nor overall survival and telomerase index. However, these associations were found when 41 rectal cancers were evaluated ($P = 0.020$ and $P = 0.010$, respectively): patients with tumours with low telomerase index showed a higher recurrence-free survival and overall survival probability. Moreover, when multivariate analysis was applied, Bautista *et al*[72] found that the telomerase index was an independent prognostic factor for predicting the recurrence in the first two years after surgery (95% CI: 1.09 to 10.8; $P = 0.030$) and for survival in rectal cancer patients (95% CI: 1.07 to 12.7; $P = 0.030$). This fact suggests a different behaviour for telomerase depending on its localization (Table 2). The findings of the present study are in accordance with other studies that identified differences in the etiologic, pathologic, and clinical behaviour of colon and rectal tumours: local recurrence occurs more frequently in patients with rectal cancer, and distant metastases occur more frequently in patients with colon cancer[73-75]. It is, therefore, reasonable to suggest that the etiologic factors and molecular bases may differ between colon and rectal cancers[72].

CONCLUSION

Gertler *et al*[43], Engelhardt *et al*[46] and Boldrini *et al*[59] support the hypothesis that sufficient (hTERT-mediated) telomere stabilization is achieved late in tumourigenesis after extensive cell proliferation and telomere shortening have already taken place. Telomere maintenance or even elongation seems to be essential for the tumour to maintain its (indefinite) proliferate capacity and to continue further tumour invasion and progression[76,77]. Effective (hTERT-mediated) telomere length stabilization might thus be a selection criterion for colorectal carcinoma to proceed from early to advanced tumour stages, illustrated by higher telomere length ratios in advanced tumours compared with early-stage tumours[45]. Moreover, telomerase evaluation may help to confirm the malignant transformation in polypoid colorectal lesions with different levels of dysplastic alterations[60].

Hahn *et al*[78] and Hahn *et al*[77,79] identified hTERT-mediated telomere maintenance as a key step in cell immortalization and neoplastic transformation of human cells and also stated that cells are selected for reactivated telomerase.

Taking into account all the results achieved by different groups, quantification and evaluation of telomerase activity and measurement of telomere length may be useful methods for additional biologic and prognostic staging of colorectal carcinoma. Moreover, the results presented by Garcia-Aranda *et al*[44] may help to identify a subgroup of patients with CRC who have a good clinical outcome among patients with telomerase positive tumours.

Gertler *et al*[43] consider that telomerase activity might be bypassed by alternative lengthening of telomeres or influenced by additional factors such as telomerase inhibitors, alternate splicing of hTERT transcripts[80], and changes of hTERT mRNA at the posttranscriptional level[81]. In agreement with that, Kawanishi-Tabata *et al*[69] propose the inclusion of other markers such as tumour suppressors or oncogenes to evaluate prognosis in CRC as clinical correlation studies based solely on telomerase activity may not be adequate. Therefore, it has been suggested that telomere length is the most reliable and most significant parameter of telomere regulation. It has the highest prognostic potential and it best defines possible candidates for new therapeutic protocols, when calculated as the ratio of cancer to non cancerous tissue[43,44,59].

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EDITORIAL

Undefined familial colorectal cancer

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Abstract

Colorectal cancer (CRC), one of the most common cancers of the world, is actually a spectrum of several subtypes, with different molecular profiles, clinico-pathological characteristics and possibly separate pathways of progression. It is estimated that in approximately 25%-35% of cases, a familial component exists, so they are classified as familial CRC (fCRC). However the known hereditary CRC syndromes justify only up to 5%. The rest are attributed to some inherited genetic predisposition passed to offspring through low-penetrance genes, which in the proper environmental setting can bring on tumorigenesis. Furthermore, part of the familial clustering may be attributed to chance. Because of the complexity regarding the etiology of CRC, the clinician is sometimes faced with obscure patient data, and cannot be sure if they are dealing with fCRC or sporadic CRC. The elucidation of what is going on with the as yet "undefined" portion of CRC will aid not only in the diagnosis, classification and treatment of CRC, but more importantly in the proper adjustment of the screening guidelines and in genetic counselling of patients.

Key words: Colorectal cancer; Familial; Undefined; Type X; Polymorphisms

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer death in the world. This is attributable to both its relationship to many environmental factors common in everyday life (especially in developed countries) and to its complex genetic background, which seems much more complicated than was once believed and is just starting to unravel.

Traditionally, CRC has been broadly classified into sporadic and inherited, the first being the result of successive spontaneous mutations and the latter being the ultimate outcome of a germline mutation that initiated the carcinogenic process. Familial adenomatous polyposis (FAP), Lynch syndrome (originally described as hereditary non-polyposis colorectal cancer or HNPCC) and several other syndromes fell in the latter category. However a significant percentage (20%-25%) of patients with CRC have some features of hereditary cancer but cannot be classified into any of the currently recognized syndromes since they don't feature the classic "single-gene defect" genotype. Considered retrospectively, it is possible that these patients have had some increased risk attributable to inherited mutations which were not sufficient to trigger

carcinogenesis on their own, and are thus put in the category of familial CRC (fCRC).

In this article, we briefly review the latest literature concerning the classification of CRC and try to explain the basis of familial CRC. Further progress on this would be valuable for genetic counseling and preventive decision making; on one hand, patients at increased risk could be advised to take the appropriate prophylactic measures, while on the other hand overdiagnosis of hereditary CRC syndromes and subsequent unnecessary actions could be avoided.

DEFINED HEREDITARY SYNDROMES AND PATHWAYS

In 1990, Fearon *et al*[1] proposed a very attractive model concerning the putative molecular events underlying the progression of colorectal cancer through several morphological stages known as the "adenoma-carcinoma sequence". Subsequent research refined this and generated the "traditional pathway" model. Approximately 80%-85% of CRCs develop through this pathway, which has also been called the chromosomal instability (CIN) or "suppressor" pathway[2].

CIN is the one of the two categories of genomic instability, and is a state in which a pre-cancerous clone has developed a cellular environment permissive of future mutation; this gives the advantage of accumulation of strategic mutations and accelerated carcinogenesis. In CIN the genetic events necessary for cancer development are favored by chromosomal abnormalities (aneuploidy). The early stages of this pathway are associated with mutations in APC (5q) and KRAS (12p) or deletions of their respective chromosome parts, while in later stages loss of heterozygosity (LOH) at the DCC/SMAD4 (18q) and P53 (17p) loci appears to be very frequent.

Adenomatous polyposis coli (APC), the gene mutated in FAP and related syndromes (Attenuated FAP, Gardner's and Turcot's syndrome), is an important regulator of growth, differentiation and apoptosis. Mutation or loss of APC may contribute to the cell's malignant potential in two ways. Firstly the Wnt cascade[3], important in maintaining the tissue-specific stem cell compartment[4], becomes more active favoring the initiation of the aberrant crypt foci (ACF). In addition to that, disturbance of β-catenin signaling, which among others participates in intercellular communication through interaction with cytoskeletal components (mainly E-cadherin), may give the cell some independence from inhibitory contact signals. Finally the APC gene is important in promoting correct chromosomal alignment and subsequent chromosomal segregation during mitosis, so that APC deficient cells can bypass the metaphase checkpoints without halting and undergoing apoptosis in case of chromosomal abnormalities[5,6].

The importance of the above is obvious from the fact that APC aberrations are found in 70%-80% of CRCs, irrespectively of whether they are familial or sporadic, while in the rest of the CRCs which retain a completely normal APC protein, mutations in other components of the Wnt pathway are found (β-Catenin, Axin, Conductin, GSK3β, TCF4 *etc*)[7]. APC is a large gene, so aberrations can occur in multiple ways, with variable effects[8]. Hence, mutations that do not completely abrogate APC's function lead to a less marked phenotype called "Attenuated FAP" (AFAP). Furthermore, mutations of MutYH - a gene encoding for a DNA glycosylase involved in base excision repair - increase the rate of G:C→T:A transversions[9]; this particularly affects the APC gene, leading to the MutYH-associated polyposis syndrome (MAP) - an autosomal recessive version of AFAP. MutYH inactivation is also associated with activating mutations of Kirsten Ras (KRAS), a proto-oncogene which encodes for a G protein that is an integral part of the Mitogen-Activated Protein Kinase (MAPK) pathway.

The remaining 15%-20% of CRCs develop through another pathway which comprises Microsatellite Instability (MSI), the second type of genomic instability. MSI is characterized by expansion or contraction of nucleotide repeat sequences which stems from dysfunction of the cell's mismatch repair (MMR) system. This can be the result of germline mutations in MMR genes (e.g. MSH2, MSH6, MLH1) as happens in Lynch Syndrome, or of epigenetic silencing through methylation of their promoters (especially of the MLH1 gene). This alternative pathway (also called the "mutator" pathway) is characterized on the one hand by frameshift mutations in critical genes with coding microsatellite sequences (such as TGFβR2, BAX, TCF4, RIZ, IGF2R) and on the other hand by a generalized tendency to point or frameshift mutations.

Through the progress made lately on epigenetics it has been established that a great fraction of CRCs are characterized by global hypomethylation of the genome and concurrent hypermethylation of cytosine bases at the promoter regions of strategic genes[10]. As a result oncogenes are switched on while tumor-suppressor genes are switched off. Hence the CpG Island Methylator Phenotype -positive [CIMP(+)] cancer was defined[11,12], calculated to occur in approximately 30% of CRCs[13]. CIMP does not perfectly overlap with MSI, but it encompasses a big part of it (Figure 1). It also partly overlaps with CIN cancers and is a feature of about half the sporadic CRCs.

The most noteworthy thing about CIMP is the fact that CIMP(+) CRCs do not usually progress through the adenomatous polyps pathway (AD) as in FAP, but instead through a novel sequence termed the serrated neoplasia pathway (SP)[14,15]. SP is mainly characterized by three alterations at the molecular level, namely activation of the MAPK pathway, inhibition of apoptosis and disturbances in DNA methylation. The first seems to occur through mutation of KRAS or BRAF (the v-raf murine sarcoma viral oncogene homolog B1) and sometimes through downregulation of the Ephrin B2 (EPHB2) gene. In fact KRAS and BRAF - two molecular switches acting

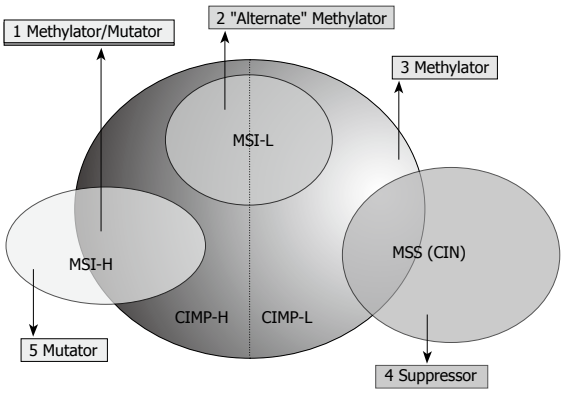

Figure 1 The spectrum of the molecular profile of colorectal cancers. Groups 1 to 5 are estimated to amount for 12%, 8%, 20%, 57% and 3%, respectively. CIN: Chromosomal instability; MSS: Microsatellite stable; MSI: Microsatellite instability; CIMP: CpG island methylator phenotype; -H: High; -L: Low.

sequentially in the MAPK pathway - appear to be mutated in an almost mutually exclusive way in CRCs.

Serrated polyps are believed to be the original lesion from which CRCs can evolve *via* the SP. They are common in the elderly and include subtypes such as aberrant crypt foci, conventional hyperplastic polyps, mixed polyps, serrated adenomas and sessile serrated adenomas (SSA), all being biologically distinct as signified by differences at the molecular level[16]. SSAs however often have an aggressive behavior and may be part of the Hyperplastic Polyposis Syndrome (HPS, proposed to be renamed to serrated adenomatous polyposis syndrome), defined by Burt and Jass[17]. They may progress through dysplasia to serrated adenocarcinoma and show a predilection for the right colon in middle-aged females. At the molecular level, they display a high level of BRAF mutation instead of KRAS. On the other hand, SSAs may represent the precursor lesion to sporadic MSI carcinomas[18].

Several hereditary forms of CRC are well recognized. The classic FAP is defined by the presence of hundreds (or more) of colorectal adenomas, which will almost certainly undergo malignant transformation in the future. Attenuated FAP and MutYH-associated polyposis are both defined by the genetic defects described above. Morphologically, they are characterized by a milder phenotype than FAP and account for approximately 15% and 35%, respectively, of a heterogeneous group of cases called multiple colorectal adenomas (MCRAs), which is defined as 5-100 adenomas lifelong.

On the other hand, the forms of CRC that were evidently hereditary but featured no adenomatous polyps (or rarely very few of them) were collectively classified as HNPCC. The classification was originally based on the Amsterdam Criteria (AC) (Table 1). However, after further advances of our understanding of molecular genetics, the original definition of HNPCC proved to be wrong, as it did not correspond to a single disease with distinct etiology[19]. The term "Lynch syndrome" is preferable. It corresponds to a hereditary cancerous disease that is explained by a germline mutation in a DNA MMR gene. The AC are traditionally used, but seem to lack both sensitivity and specificity. The Bethesda guidelines (Table 1) are more sensitive and may be used for choosing whom to test for MMR deficiency, which adds to specificity as well[19,20].

Table 1 Amsterdam Ⅰ and Ⅱ criteria and Bethesda (revised) guidelines

Term	Criteria
Amsterdam Ⅰ[65]	At least three 1st degree relatives with CRC
(All criteria must be met)	At least two successive generations affected
	At least one family member diagnosed below age 50
	FAP excluded
Amsterdam Ⅱ[65]	As for Amsterdam Ⅰ except that CRC may
(All criteria must be met)	be substituted by any of: CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis
Bethesda (revised)[66]	Test for MSI if: (Any criterion met)
1	CRC diagnosed below age 50
2	Presence of multiple CRC or HNPCC-related cancers[1] (synchronous or metachronous)
3	CRC with MSI-H-related histology[2] diagnosed below age 60
4	CRC or HNPCC-related cancer in at least one 1st degree relative, diagnosed below age 50
5	CRC or HNPCC-related cancer in at least two 1st or 2nd degree relatives, regardless of age

[1]Includes cancer of endometrium, small bowel, pelviureter, biliary tract, stomach, ovary, pancreas, and brain (mainly glioblastoma multiforme); [2]Tumor infiltrating lymphocytes, Crohn-like reaction, mucin/signet ring cell differentiation, medullary growth pattern.

Molecular-histological correlation

The recent trend has been to classify CRC according to molecular features, which are a direct consequence of the carcinogenic pathways involved in each case. A scheme proposed by Jass *et al*[21] involves five subtypes, based primarily on (1) the underlying types of genetic instability, and (2) the presence of DNA methylation. Certain clinical (location, gender) and pathological (serration, precursor lesion, tumour infiltrating lymphocytes, dirty necrosis) features interestingly fit quite well to certain subtypes. These groups may be conceived as completing a circle, rather than representing a continuous spectrum, as Jass says. The proposed classification may assist in recognition of familial cases and formulation of more appropriate criteria for fCRC, as well as effective screening of at risk patients.

Microsatellite (MS) status is graded according the Bethesda panel, which divides CRCs into three categories: MS stable (MSS) if none of them is changed, low MSI (MSI-L) if there is alteration in one of the five and high MSI (MSI-H) if two or more are altered. MSI-L CRCs show higher rates of KRAS mutation. Moreover there is very frequent methylation of the promoter of O-6-methylguanine-DNA methyltransferase (MGMT), which constitutes the genome prone to transversions and may be the mechanism to the KRAS point mutations.

Methylation status can be assessed using several panels. CIMP-positive CRCs have been subdivided into

CIMP-high (CIMP-H) and -low (CIMP-L). CIMP-H CRCs frequently harbor BRAF mutations and have a generalized increase in de novo methylation. CIMP-L CRCs almost always have KRAS mutations and a denser but less widespread pattern of methylation involving fewer genes. CIMP-H status correlates much more strongly with a positive family history of CRC.

Group 1 [CIMP-H, MSI-H, BRAF mutation, CIN(-), methylation of MLH1] or "Methylator/Mutator" is believed to originate in serrated polyps and represents the sporadic MSI-H CRCs.

Group 2 [CIMP-H, MSS or MSI-L, BRAF mutation, CIN(-), partial methylation of MLH1] or "Alternate methylator" also originates in serrated polyps. Here, MGMT loss may also occur and a synergistic effect with the loss of expression of MLH1 is possible. This group could be regarded as a "group 1/group 4 hybrid".

Group 3 [CIMP-L, MSS or MSI-L, KRAS mutation, CIN(+), MGMT methylation] or "Methylator" may originate in villous adenomas, in serrated polyps, or in mixed polyps. Along with group 2, it represents the intermediate between the MSI-H and the MSS/CIN(+), and a big part of the fCRC may fall in these two groups.

Group 4 [CIMP(-), MSS, CIN(+)] or "Suppressor" may be sporadic, FAP-associated or MAP-associated and constitutes the biggest part of the pie (approximate 57%). It evolves through the traditional adenoma pathway and APC mutation is the hallmark of this group.

Finally, group 5 [CIMP(-), MSI-H, CIN(-)] or "Mutator" originates in adenomas and is actually the Lynch syndrome, i.e. the familial MSI-H CRC.

The interrelationship between these groups and their respective characteristics are depicted in Figure 1.

UNDEFINED FAMILIAL CANCER

General perspective

The term familial cancer is not absolute. It is defined according to several panels such as the AC, which were arbitrarily set up and are mainly based upon the family history of cancer. Therefore, the percentage of familial CRCs varies according to the definition. Although twin studies report inherited susceptibility to amount at approximately 25%-35% of CRCs, the classic genetic syndromes that fit the Mendelian inheritance [FAP, Lynch syndrome, Familial juvenile polyposis, PTEN-associated polyposis (Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome) and Peutz-Jeghers syndrome] are quietly rare and account for only 3%-5% of the total[22]. The remaining 20%-30% are currently attributable to various combinations of low-penetrance mutations, which either function as modifier genes or act in concert with environmental factors to initiate and enhance the carcinogenic sequence[23]. In fact, such mutations are highly likely to be responsible for the biggest part of familiality of CRC, since individuals with high-penetrance mutations would tend to be extinguished through negative physical selection. In support of this stands the fact that there are no recognized high-penetrance genes for cancers with documented high familiality (such as thyroid cancer) evident by twin studies. This notion has been called the "common disease - common variant" hypothesis[24].

MutYH issue

As mentioned above, mutations of the MutYH gene result in the recently recognized syndrome of MutYH-Associated Polyposis (MAP), with a phenotype similar to - or even indistinguishable from - FAP and especially AFAP[25]. The hallmarks of MAP are biallelic germline mutations of the MutYH gene and hence it is inherited as an autosomal recessive disorder. These patients develop a variable number of polyps and carry significant risk of progression to CRC, as evidenced by several studies. In fact up to one quarter of cases with the FAP/AFAP phenotype who are negative for APC mutations are found to have biallelic inactivation of MutYH. Therefore it is prudent that such suspicious cases be tested for their MutYH status and if found positive, then managed with at least an appropriate screening program, such as colonoscopy every one or two years.

A reasonable question that arose is what happens with monoallelic germline mutation carriers? This question has posed considerable debate among researchers, with contradictory results occurring from several series[26,27]. The monoallelic effect seems to be of borderline statistical significance and the inconsistencies probably stem both from bias issues and from the smallness of samples, which as a result are not enough to demonstrate the effect of low-frequency mutations. Hence there is urgency for a large scale study with sufficient power. Of note, a meta-analysis of several case-control studies by Jenkins *et al*[28] showed that monoallelic carriers of MutYH do not manifest MAP but are at a 3-fold increased risk of CRC and have a cumulative risk of 8% of developing CRC by the age of 70.

Hyperplastic polyposis syndrome

Hyperplastic Polyposis Syndrome (HPS) has been reported to cluster in families and CRC occurs in the relatives of HPS individuals very often[29,30]. It is postulated to be inherited in a co-dominant mode, so in the case of heterozygotes, it may have varying phenotype, depending on the nature of the second allele. This would partly explain the role of putative alleles, which although innocuous on their own, could be detrimental in the proper setting. It would also account for part of the burden of SP CRC in the population. Although CIMP positive, carcinoma in HPS is more likely to be non-MSI high.

CRC in the context of HPS occurs through the SP, at least in a fraction of patients[16]. An interesting yet complex relationship seems to exist between the molecular mechanisms. Although KRAS or BRAF mutations occur early in the setting of HPS, they are not protumorigenic on their own and may lead to replication arrest and even apoptosis. However, in the presence of CIMP, the situation is reversed. This probably results from the silencing of critical genes implicated

in apoptosis and cell cycle control through promoter methylation. An underlying mechanism directing the methylation of genetic loci that bear specific motifs has been suggested[31]. A vicious circle then ensues where genetic alterations accumulate and methylation patterns are disturbed, the cell becomes even more independent of growth-inhibitory and apoptotic signals and so on.

What is special then about HPS patients? Apparently they have an inherited tendency to CIMP, which creates a field cancerization effect upon their colon (and possibly other organs) through inappropriate hypermethylation[32]. CIMP acting in synergy with a somatically acquired BRAF mutation opens the door to CRC *via* the SP. The process is compounded by environmental exposure; smoking in particular has been proven to significantly increase the risk for CRC in the presence of CIMP and BRAF mutation[33].

"Familial Colorectal Cancer Type-X" syndrome

Lindor *et al*[34] introduced the term "Familial Colorectal Cancer Type-X" to encompass all the CRC incidents with evidence of familiality based on any number of pedigree and/or laboratory criteria (including the AC) that are not Lynch Syndrome (i.e. do not have any evidence of MMR deficiency). This is not an actual disease entity, but rather a group that includes all the familial non-MMR-mutated CRCs, which was devised to compensate for our lack of understanding for the exact etiology, hence the "X". In a recent article, Jass explains why HNPCC is an unfortunate term and further clarifies the application of fCRC Type-X[19]. However fCRC "type X" patients have a lower incidence of CRC than actual Lynch syndrome patients, while the risk for extra-colonic cancers may not be increased at all. Moreover, the age of onset of CRC may be significantly different, since Lindor *et al*[34] found the mean age of diagnosis to be greater by 12 years in the "type X" group. Hence a patient should not be loosely tagged as "type X" if not all the hereditary CRC syndromes have been excluded. The checklist should also include conditions like Juvenile Polyposis, Hereditary Polyposis Syndrome *etc*, which, although rare, are attributable to specific mutations and have specific pathologic characteristics[35].

A big part of the multiple colorectal adenomas (MCRA) group mentioned before is not explained by the as yet defined syndromes (AFAP/MAP), but family history of CRC is present. Therefore it may fall under the fCRC Type-X heading. Currently, this is attributed to germline variation, which can accelerate carcinogenesis under the appropriate circumstances. A very recent study on German patients of this category (i.e. family history in the absence of a recognized CRC syndrome) found increased prevalence of MutYH mutations, compared to sporadic CRC or controls[36]. This finding, together with the results of the meta-analysis mentioned above[28], imply that inactivation/loss of one MutYH allele may be responsible for part of the MCRA hereditary predisposition to CRC. Variations in genes of the Wnt pathway may also account for part of the MCRA, since β-catenin is significantly overexpressed in these patients[37].

Of course we must not overlook the possibility of part of the familiality of CRC in the "X" syndrome being attributable to common environmental exposure. Several studies have shown that in various settings there are strong associations between shared lifestyle factors (e.g. smoking and alcohol intake) and family history of CRC[38]. Hence, in some cases, family history may actually be a confounding factor, while the real culprit for increased CRC risk is the shared environment. However these studies have many inconsistencies as well as many limitations regarding the patients' characteristics (gender, age, *etc*), warranting further evaluation of their results.

Upon recognition of a suspicious pedigree, the key clinical question to be answered is whether we are dealing with a hereditary cancer or several random sporadic events. This will guide the management of both the patient and his asymptomatic relatives. Answering this question is not feasible in many cases, because the causative mutation is not known and hence the desirable genotypic investigation cannot be done. However, the common disease - common variant hypothesis implies that if we find a way to screen for the low-penetrance mutations, then maybe we will be able to predict - at least partly - the risk of individuals of developing CRC. Among others, this could potentially contribute to increasing the compliance of patients to lifestyle modifications as preventive measures.

Since low-penetrance mutations usually take the form of single nucleotide polymorphisms (SNPs), the desired analysis may be accomplished by conducting genome scans for SNPs. A prerequisite for this is to recognize SNPs that confer increased risk for CRC, and at a later stage to quantify the risk and find the best-benefit treatment.

Role of polymorphisms

Lately much research is being conducted on discovering novel low-penetrance mutations, with quite a few definitive and in some cases with confusing results. One reason for that is the use of association studies (a type of case-control study), which do not have sufficient power. As a result, some eventually prove to be inaccurate, since they cannot be reproduced in subsequent studies. For example the much-mentioned TGFβR1 × 6A variant of the TGF-β type 1 receptor has been found in several studies to be responsible for a slightly increased risk for CRC (20%) which is also dose-dependant (homozygotes > heterozygotes) and is important because it is quite common in the general Caucasian population, raising the attributable risk to 1.2%[39,40]. However a very recent study on 1042 CRC cases *vs* 856 controls found the relative risk to be only 1.05, which was not significant and concluded that there is no association of the polymorphism with increased CRC risk[41].

An interesting point is that several suggested polymorphisms result in small repeats of a nucleotide. Such oligonucleotides are prone to replication errors through DNA polymerase slippage and constitute a form of genomic instability. The most thoroughly studied polymorphism is probably the I1307K of the APC

gene, found in a significant percentage of the Ashkenazi Jews[42]. This change creates a stretch of eight adenines (A8) instead of the normal A3TA4, which is vulnerable to insertions or deletions that create a truncated APC protein. A second polymorphism in the APC, the E1317Q, may increase the risk for MAP, although still unproven. Given the crucial role of the Wnt pathway in colorectal carcinogenesis - which is virtually always deregulated in CRCs - we should expect polymorphisms affecting genes encoding for other molecules of the cascade also to modify the risk[8].

Many other gene polymorphisms have been suggested as candidate low-penetrance mutations. They correspond to genes participating in many different functions, including metabolism, cell cycle control, maintenance of DNA integrity and immune response. Polymorphisms in glutathione-S transferase theta (GSST1) and N-acetyl transferase (NAT2) genes impact on the biotransformation of carcinogenic substances, possibly resulting in decreased detoxification and increased presentation of carcinogens to crypt cells[43,44]. The enzyme 5,10-methyletetrahydrofolate reductase plays a pivotal role in the metabolism of nucleotides and thus in the repair of DNA errors. Homozygotes for valine at position 667 (MTHFR × 667V) have a protective advantage, because the decreased levels of methylene-THF interfere with thymidylate biosynthesis, leading to deoxyuridylate pool imbalances[45]. A similar effect was found recently with homozygosity for valine at position 222 (MTHFR × 222V)[46]. However, a newer study in the Japanese population demonstrated that haplotypes that lead to reduced MTHFR activity are associated with promoter hypermethylation and subsequent increased risk of CIMP(+) CRC[47]. The latter finding is supported by the observation that systemic DNA hypomethylation may occur in the case of inadequate dietary folate supplementation, which is at least partially reversible when folate intake is restored to physiologic levels[48,49]. The whole case here represents a nice example of the intricate interaction between genetic polymorphisms and environmental exposure, as well as how strikingly different the inter-population variations can be.

Genes involved in DNA repair comprise another category believed to modify the risk for CRC through subtle changes in their sequences[50]. Many polymorphisms in several genes have been proposed as candidates[51] but few are proven to affect the risk in the general population. Only in the case of OGG1 and XRCC1, involved in base excision repair, and XPD for nucleotide excision repair, consistent evidence exists for association with certain types of cancer. Furthermore, the study of Webb and Rudd mentioned above raises the possibility of polymorphisms in the genes ATM and CHEK2 predisposing to CRC, through alterations in the axis ATM-CHEK2 that regulates cell cycle checkpoints based on signals for DNA integrity[46].

The gene BLM encodes a homologue of recQ helicase, biallelic inactivation of which causes genomic instability and ultimately leads to Bloom syndrome. Heterozygotes for a particular frameshift mutation, the BLMAsh (2281 delATC TGA insTAG ATT C) in exon 10, are at approximately 2.5-fold increased risk for CRC[52]. Although heterozygotes do not appear to have increased familial clustering attributable solely to BLMAsh, it is possible that this mutation modifies the risk conferred by other variants, such as the APC × I1307K (both found in Ashkenazi Jews), and hence indirectly affects the predisposition to fCRC[53].

Another recent study confirmed the previously reported increase in risk arising from polymorphisms in the Cyclin D1 gene (CCND1) - a cell cycle control protein - and E-cadherin (CDH1), an intercellular adhesion molecule[54]. In particular, the A→G mutation in position 870 of CCND1 and the C→A variant at codon -160 of CDH1's promoter were found at increased frequency in fCRC cases, compared to sporadic cases[55]. On the contrary, this study failed to demonstrate any association between fCRC and variants of the TP53, vitamin D receptor (VDR) and ileal bile acid transporter (SLC10A2) genes. However that may not be the story with CCND1, since a study in Singapore showed that the effect of the A870G polymorphism may not act on its own, because it is modified by polymorphisms in glutathione S-transferases as well as isothiocynate intake[56]. This is another proof of how cancer risk is based on a combination of genetic predisposing factors which interact with environmental stimuli to bring on carcinogenesis.

Harvey Ras (HRAS1) is a proto-oncogene with an accompanying variable number tandem repeat (VNTR) minisatellite downstream. Polymorphisms in the latter (HRAS1 × VNTR) were shown to interact with molecules of the NF-κB family as well as other transcription regulators and hence modulate the expression of nearby genes. Some rare alleles appear to increase the risk for CRC, although this may also result from linkage disequilibrium[57].

Recent evidence also correlates SMAD7 polymorphisms with increased CRC risk[58]. SMAD7 acts as an intracellular antagonist of TGF-β signaling, deregulation of which has been shown to influence CRC progression[59,60]. Moreover, these polymorphisms are postulated to interact with other common alleles to substantially increase an individual's risk. SMAD7 status is being investigated as a tumor marker for CRC, since its amplification is associated with poorer prognosis.

Finally, polymorphisms in inflammatory response-related genes may modify the CRC susceptibility. Inflammation seems to favor tumorigenesis through various mechanisms, such as sustained DNA damage, stimulation of cell proliferation and provocation of angiogenesis. Considering that the colonic mucosa is naturally in a state of continuous inflammation because of the normal flora, factors that would aggravate this state could tilt the balance towards carcinogenesis. In a study conducted on a sample from the Greek population by our team, a significant association was found between polymorphisms of some inflammatory response-related genes and increased CRC risk[61]. Specifically, the R241G

and K469E allelic variants of ICAM-1, as well as the -174G of IL-6 increase the risk of CRC, probably *via* the aforementioned mechanism. The GG genotype at -174 of IL-6 further increases the risk. The CC genotype encoding for proline at position 12 (Pro12) of the PPARγ gene was also correlated with increased CRC susceptibility. On the other hand the Ala12 variant of PPARγ was found by others to have a protective effect. The effects of these polymorphisms may be related to the fact that PPARγ can alter the expression of COX-2. In addition to that, PPARγ can regulate the transcription of the tumor suppressor gene PTEN and hence modify several signaling pathways.

CONCLUSION

Colorectal cancer is very common nowadays. The once thought single disease is actually a spectrum of several subtypes, with different molecular profiles, clinico-pathological characteristics and possibly separate pathways of progression. New data concerning the molecular pathways of colorectal cancer evolution and the histopathology of the precancerous lesions are coming to light every day. These are expected to have decisive implications not only in the diagnosis, classification and treatment of CRC, but more importantly in the adjustment of screening guidelines in order to catch the disease early, in a perhaps curable stage.

Family history remains fundamental in the diagnosis, management and prognosis of CRC, but also in counseling and preventive interventions. However it should not be the sole guidance to diagnosis of hereditary syndromes, but should always be considered in a broader context together with clinical, pathological, molecular and biological characteristics of the tumor. The possibility of familial aggregation due to shared environment and even chance should always be kept in mind. Because of that, the family history of patients should be thoroughly obtained, but no family should be tagged as having a hereditary cancer syndrome if no solid evidence (supported by pathology and molecular features) exists[62].

The emerging concept of SNPs modifying the risk of CRC seems fascinating. The inconclusive or controversial data regarding their role may suggest that the modulation of cancer risk depends on a joint effect of multiple polymorphisms within different genes or pathways, interacting with environmental factors. In order to confirm the putative role of SNPs as low-penetrance mutations, large-scale, genome-wide linkage studies have to be conducted, using highly efficient analytical platforms and proper stratification. These will overcome most of the bias, will be much less likely to miss important variants and will thus have much more power. One such multi-center trial is currently in progress in the UK and aims to gather and analyze 20 000 CRC cases[63].

MutYH-associated polyposis is just beginning to be elucidated. Screening for MutYH mutations in cases of polyposis phenotype with negative APC mutations may have special implications in the management and follow-up, as well as on the genetic counseling of relatives, especially of siblings[64].

Finally, the need for a review of the guidelines on screening and management of patients with positive family history of CRC will not be long to come. Having ruled out all the known hereditary CRC syndromes, while being confident about the familial component, the clinician has possibly discovered another "type X" patient. Based on the less severe phenotype of this entity, these patients must be treated with less aggressive measures than actual Lynch syndrome patients, such as screening every five years but no prophylactic surgery.

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OBSERVATION

Concept of chemoprevention in colorectal cancer

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Abstract

Colorectal cancer remains a significant cause of morbidity and mortality throughout the world. The incidence of colorectal cancer is nearly four-fold higher in more-developed as compared with less-developed regions of the world. At present an early detection of colorectal cancer remains a crucial step in determining the therapeutic outcomes. Screening programmes have been introduced in an effort to detect colorectal cancer at an early stage or at a precancerous colonic polyp stage. These programmes should be used by the health professionals as an opportunity to educate the public regarding the use of chemoprevention in colorectal cancer, which is the main focus of this review and an attractive concept needing further evaluation.

Key words: Colorectal cancer; Chemoprevention; Geographical variations; Dietary carcinogens

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide leading to significant mortality and morbidity[1]. Colorectal cancer incidence rates both among men and women are nearly fourfold higher in more-developed as compared with less-developed regions of the world[1]. The reasons for this variability in CRC prevalence in different geographical areas are not fully known. This however has been studied extensively and the role of various carcinogenic factors has been the target of clinical, experimental, and epidemiological studies. Most studies favour an aetiological role of dietary, lifestyle, and genetic factors in CRC, however, some studies have demonstrated equivocal or even negative results. These observations have been analysed in detail in a recent review[2].

Colorectal screening programmes are an excellent opportunity to educate the public regarding the carcinogenic potential of dietary and lifestyle factors. Current emphasis of most CRC screening programmes is to detect cancer at an early, or preferably at a precancerous-stage of colonic polyps. This approach maximises survival outcomes. Modifications of carcinogenic factors coupled with chemoprevention are important targets for the future approach to CRC prevention. We will focus on the role of chemoprevention in this review, while giving brief description of lifestyle and dietary factors.

CHEMOPREVENTION IN COLORECTAL CANCER

Observations from earlier experimental studies suggested a protective effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the development of tumour growth[3,4]. Such experimental data was supported from epidemiological studies which showed significant

Table 1 Use of aspirin in average risk subjects and colonic neoplasia risk reduction

Study	*n*	Aspirin dose (mg/AD)	Study duration (yr)	Relative risk reduction (95% CI)
Thun *et al*[13]	622 424	100 mg[3]	⩾ 1	0.58 (0.36-0.93)[1]
				0.61 (0.38-0.97)[2]
Gann *et al*[14]	22071	325	5	1.15 (0.80-1.65)[1]
Cook *et al*[15]	39876	100	10	0.97 (0.77-1.24)[2]
Stürmer *et al*[16]	22071	325	12	1.03 (0.83-1.28)
Giovannucci *et al*[17]	47900	325	4	0.54 (0.34-0.84)[1]
Paganini-Hill *et al*[18]	13979	325	7-10	1.38 (NR)[1]
Chan *et al*[19]	89446	100	10	0.62 (0.44-0.86)[2]
Friis *et al*[20]	29470	325	6	0.9 (0.70-1.10)[1]
García-Rodríguez *et al*[21]	12005	325	> 2	0.9 (0.8-1.1)
Reeves *et al*[22]	845	100	> 5	0.79 (0.46-1.36)[2]
Juarranz *et al*[23]	502	325	NR	0.32 (0.09-1.10)
La Vecchia *et al*[24]	3248	325	5	0.7 (0.50-1.00)
Kune *et al*[25]	1442	325	NR	0.57 (0.41-0.79)
Suh *et al*[26]	2704	325	6	0.33 (0.15-0.72)
Slattery *et al*[27]	3051	325	> 5	0.7 (0.6-0.8)

[1]Males; [2]Females; [3]Daily; AD: Alternate day dosage; CI: Confidence interval; NR: Not reported.

Table 2 Role of aspirin in colorectal neoplasia risk among patients with past tumour

Trials	*n*	Aspirin dose	Duration (yr)	Relative risk (95% CI)
Baron *et al*[28]	1121	81/325 mg	1	0.96 (0.81-1.13)
Benamouzig *et al*[29]	272	160/300 mg	1	0.82 (0.7-0.95)
Greenberg *et al*[30]	864		4	0.52 (0.31-0.89)
PPSG[31]	1905	< 325 mg	4	0.82 (0.65-1.02)
		> 325 mg		0.54 (0.3-0.96)
Sandler *et al*[32]	492	> 15 tab/month	5	0.84 (0.5-1.43)
Breuer-Katschinski *et al*[33]	442	> 4 tab/week	< 5	0.91 (0.32-2.64)
			> 5	0.09 (0.01-0.82)

CI: Confidence interval; PPSG: Polyp prevention study group.

nonspecific tumour risk reduction of between 20%-50% among NSAID users[5-7]. A consistent beneficial effect of aspirin and NSAIDs in reducing formation of colonic polyps was also reported in a systemic review[8].

Cancer chemopreventive mechanisms for NSAIDs

It is suggested that colorectal carcinoma risk reduction from NSAIDs is mainly related with inhibition of cyclooxygenase (COX), particularly COX-2, which is raised in colorectal neoplasia[9]. Other effects of aspirin on the oncogenic Wnt/β-catenin pathway activity in colorectal cancer cell lines have been studied. For example a dose-dependent decreased activity of this pathway, as judged by TCF-driven luciferase activity, reduced Wnt target gene expression and increased phosphorylation of β-catenin by immunoblotting[10] has been demonstrated. In this study, ubiquitination and cytoplasmic levels of β-catenin were assessed by immunoblotting, and the localization of β-catenin was shown by green fluorescent protein-tagged β-catenin and time-lapse fluorescent imaging. Interestingly, aspirin treatment caused increased phosphorylation of protein phosphatase 2A (PP2A), an event associated with inhibition of PP2A enzymatic activity. This was confirmed by a reduction in enzymatic PP2A activity. Moreover, this inhibition of PP2A enzymatic activity appeared essential for the effects of aspirin on the Wnt/β-catenin pathway as shown by transient transfection with PP2A constructs. The findings in this trial provided a molecular explanation for the efficacy of aspirin in chemoprevention of colorectal cancer and showed biochemical evidence that PP2A had an important regulatory effect on Wnt/β-catenin pathway activity in these cells.

Chemopreventive agents and outcomes

In various trials where aspirin and NSAIDs were used as chemoprotective agents, a higher dose had favourable results, however findings were inconsistent[5,11,12]. In general the regular use of aspirin appears to reduce the incidence of colorectal adenoma with relative risk reductions on the order of 13% to 28% in average-risk individuals as shown in Table 1[13-27]. On the basis of a limited number of studies, the relative risk reductions for individuals with a history of colonic adenoma are probably higher than for those at average risk. Furthermore, it appears that longer duration of aspirin use, as well as higher doses, are associated with greater relative risk reductions than smaller doses and shorter duration as shown in Table 2[28-33]. A randomised, placebo-controlled trial included 2586 patients with a recent history of adenoma[34]. These patients were assigned to receive either placebo or selective COX-2 inhibitor (rofecoxib 25 mg/d) with results showing

significant reduction in adenoma recurrence ($P < 0.0001$) in experimental group. However, in the rofecoxib group a significantly higher rate of upper gastrointestinal and thrombotic cardiovascular event were observed. With this initial and subsequent reporting of significant cardiovascular events, use of COX-2 inhibitors as a chemopreventive agent has largely been discontinued[34,35].

5-aminosalicylates (ASA) in colorectal cancer prevention

Chemopreventive role of 5-ASA in colorectal carcinoma has been proposed in patients with inflammatory bowel disease. The effect of 5-ASA on the Wnt/β-catenin pathway has been studied in colorectal cancer cell lines to find a molecular basis underlying its chemopreventive features[36]. 5-ASA targets the Wnt/β-catenin pathway in adenomatous polyposis coli mutated cells with intact β-catenin, judged by luciferase reporter assays. In addition, 5-ASA treatment leads to reduced expression of nuclear β-catenin and Wnt/β-catenin target genes, and increased β-catenin phosphorylation. Such effects on the Wnt/β-catenin pathway are mediated *via* protein phosphatase 2A (PP2A) and increased phosphorylation of PP2A after 5-ASA treatment coincides with decreased PP2A enzymatic activity. The inhibition of PP2A enzymatic activity by 5-ASA appears to be essential for its effect on the Wnt/β-catenin pathway, as shown by transient transfection with siPP2A and mutant PP2A. These effects of 5-ASA are observed in similar doses as used in the treatment of inflammatory bowel disease.

LIFESTYLE AND DIETARY FACTORS

Lifestyle and dietary constituents including fibre content and its source, protein and fat types and their origin, and their consumption patterns vary enormously in different geographical areas and had been linked to CRC aetiology. The potential role of various dietary factors in CRC carcinogenesis and possible preventive strategies are given in Table 3.

The cancer protective role of fibre has been attributed to its bulking effect, faecal dilution factor, shortening of faecal transit time and fermentation properties. Fibre fermentation products have been studied extensively and among the various products, butyrate a naturally occurring fatty acid was found to be most relevant. Butyrate has a potential to inhibit cell proliferation, induce apoptosis and differentiation, and increase phase Ⅱ enzyme activities in tumour cells, whereas little information is available on its protective effect in less-transformed colon cells[37,38]. Butyrate from wheat bran leads to higher concentrations in distal large bowel and is more protective compared to butyrate from guar gum and oat bran[39].

The role of fat components depends on the fatty acid composition of food. Thus docosahexaenoic acid which is rich in certain fish may have a role through inhibition of the arachidonic acid cascade involved in carcinogenesis and cell proliferation[40]. The higher prevalence of colon cancer in South Africans whites (17:100 000) was investigated by estimating epithelial proliferation differences in the black Africans (cancer prevalence 1:100 000) based on dietary differences[41]. The lower prevalence of CRC in Mediterranean countries may be related to the use of extra virgin olive oil.

Pinoresinol-rich extra virgin olive oil extracts have potent chemopreventive properties and specifically upregulate the ATM-p53 cascade[42].

Data in relation to the use of red meat and a higher risk of colorectal cancer is relatively consistent, although controversies do exit[43-45]. The proposed mechanisms involved in CRC carcinogenesis and meat consumption relate to intake of a higher quantity of red meat (> 120 g/d), formation of heterocyclic amines, polycyclic aromatic hydrocarbons (dependent on cooking methods) and nitrates, N-Nitroso compound formation, and heme component[46-52].

A higher risk of CRC was suggested from a review of ecological studies which analysed meat consumption patterns among the included populations[53]. Three meta-analyses which included 15 prospective studies on red meat, 14 prospective studies on processed meat, 18 case-control studies and 19 cohorts showed colorectal cancer risk with meat consumption[54-56].

Certain lifestyle factors have been implicated in CRC carcinogenesis, including smoking, alcohol consumption, exercise lack, obesity, and genetics. Factors involved in DNA methylation, synthesis, and repair and factors with antioxidant properties may be involved in colorectal cancer risk which in turn may be influenced by other factors. In a large European cohort, both lifetime and baseline alcohol consumption showed increased colon and rectal cancer risk, with apparent risk being higher for alcohol intake greater than 30 g/d[56]. In another large prospective European multicenter study 368 277 subjects were evaluated using various anthropometric measurements, which found body weight and body mass index to be associated with a significantly higher risk of colon cancer[57]. An Austrian population-based study found an inverse association of weight loss to colorectal carcinoma while adjusting for smoking, occupational group, blood glucose, and body mass index at baseline in

Table 3 Colorectal cancer and the potential carcinogenic role of various factors

CRC category (% diagnosed with CRC)	Intervention strategies
Sporadic CRC (75%)	Screening Programmes
	Detection and removal of polyps (age > 50 yr)
	Role of chemoprevention
	NSAIDs/Aspirin
	5-aminosalicylate
	Dietary and lifestyle factors
	Good: low protein, high fibre, low fat, micronutrients, exercise
	Bad: alcohol, tobacco, obesity
Familial syndromes + miscellaneous (25%)	Screening colonoscopy
	Other: Aspirin and NSAIDs, 5-ASA

CRC: Colorectal cancer; NSAIDs: Non steroidal anti-inflammatory drugs; 5-ASA: 5-aminosalicylates; IBD: Inflammatory bowel disease.

over 65 000 subjects[58]. Consumption of micronutrients including vitamin B6, folate, calcium, selenium, caffeine has also been studied in CRC carcinogenesis with mainly controversial results[59,60].

CONCLUSION

Colorectal cancer is an important health issue particularly in the affluent countries. Chemoprevention is an attractive concept in colorectal cancer prevention. This however should be coupled with modification of other lifestyle and dietary factors which have important carcinogenic potential as evident from the current clinical, experimental, and epidemiological studies. We recommend that health professionals should promote public awareness regarding the aetiological role of the modifiable factors alongside the primary prevention using CRC screening programmes. Chemoprevention in the form of NSAIDs and 5-aminosalicylates has a significant role in individuals particularly those with genetic and other CRC predispositions.

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GUIDELINES FOR BASIC RESEARCH

Potential of casein kinase I in digestive cancer screening

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Abstract

Casein kinase I is a group of ubiquitous Serine/Threonine kinases that have been implicated in both normal cellular functions and several pathological conditions including Alzheimer's disease and cancer. Recent findings in colon and pancreatic cancer have brought tremendous attention to these molecules as potential therapeutic targets in treatment of digestive cancers. In this review, we summarize up to date what is known about this family of kinases and their involvement in carcinogenesis and other pathological conditions. Our emphasis is on their implications in digestive cancers and their potential for cancer screening and therapy.

Key words: Casein kinase I; Colon cancer; Pancreatic cancer; Gastric cancer; Biomarkers

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INTRODUCTION

Casein kinase I (CKI) family is a group of monomeric serine/threonine protein kinases that are ubiquitously found in all eukaryotic organisms. Seven members (encoded by distinct genes) have been identified so far in mammals: α, β, γ1, γ2, γ3, δ, and ε. Other eukaryotes seem to have more CKI proteins, for example, *Drosophila* has 8 and *Caenorhabditis elegans* has 87[1,2].

CKI family members are involved in many diverse and important cellular functions, such as regulation of membrane transport, cell division, DNA repair, circadian rhythms, apoptosis and cellular differentiation[3,4]. Mutations and deregulation of CKI expression and activity has been linked to various diseases including neurodegenerative disorders such as Alzheimer's and Parkinson's disease, sleeping disorders and cancer. Recent findings in some digestive cancers provide additional evidence about their critical roles in carcinogenesis and their potential utilization in cancer prevention and therapy.

PREVELANCE OF DIGESTIVE CANCERS IN THE UNITED STATES

According to the data from the American Cancer Society[5], about 19% of cancer incidence in the United States takes place in the digestive system. Among various digestive cancers (i.e. esophageal cancer, gastric cancer, intestinal cancer, colorectal cancer, liver cancer, gallbladder cancer, pancreatic cancer, *etc*), colorectal cancer represents > 50% and therefore is the most prevalent one. Despite the high numbers, both incidence and mortality rates for colorectal cancer have been declining steadily since 1975. These declines are mostly thanks to the powerful screening, which makes it possible to remove polyps before they become malignant, or to remove cancerous cells before they develop metastases[5]. Pancreatic cancer

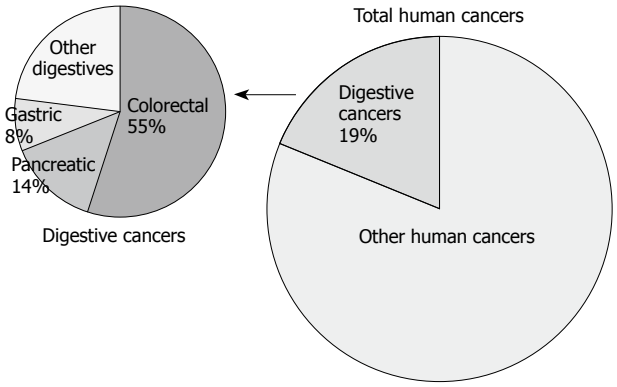

Figure 1 Prevalence and distribution of digestive cancers in USA population. Digestive cancers make up 19% of all cancers in the United States. Colorectal cancer, pancreatic cancer and gastric cancer are the three most common and/or most deadly among them.

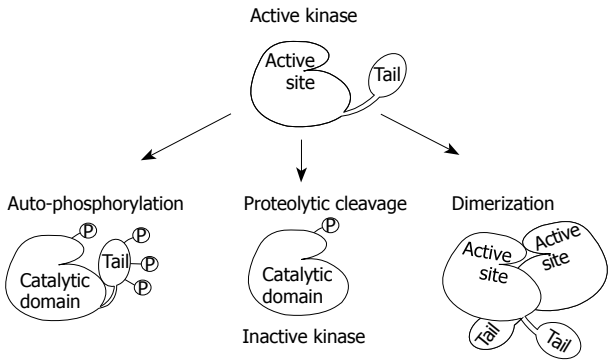

Figure 2 Models of CKIε/δ regulation. CKIε/δ auto-phosphorylation at their C-terminal tail inhibits their kinase activity through a conformational change that places the tail over the active site, therefore blocking it from potential substrates. Dephosphorylation by phosphatases is the most accepted mechanism for their activation *in vivo*. In addition, removal of the tail by proteolytic cleavage or mutations has been used *in vitro* to activate these kinases; however, actual removal of the tail *in vivo* may have additional effects on proper function. Finally, CKIε/δ dimerization at the active site could inactivate these kinases by physically blocking substrates from entering this region.

is another most deadly digestive cancer, with 34 290 expected deaths in the United States alone for 2008. According to the annual report from the American Cancer Society, only 5% of patients have 5-year survival rate[5]. This is largely due to an insufficiency of early diagnoses and a high resistance to chemotherapy. The comparison between these two cancers in their survival rates signifies the importance of identifying biomarkers that can help in the screening process as well as in the development of therapeutic strategies. Recent findings on CKIε and CKIδ in colon, pancreatic and gastric cancer, which together comprise the three most common digestive cancers in the United States (Figure 1), suggest that the CKI members may hold promise both as screening markers and as therapeutic targets.

BIOCHEMISTRY AND REGULATION OF CKI

The CKI family members have the highest homology in their kinase domains (53%-98% identical) and differ from most other protein kinases by the presence of the sequence Serine-Isoleucine-Asparagine instead of Alanine-Proline-Glutamate in kinase domain Ⅷ[6]. Outside their kinase domains, CKIα and CKIβ are 76% identical, while the CKIγ isoforms are approximate 50% identical in their C-terminal tails. CKIδ and CKIε have long C-terminal extensions with approximate 53% identity.

The CKI kinases appear to have similar substrate specificity *in vitro*[7], and substrate selection is thought to be regulated *in vivo via* subcellular localization and docking sites in specific substrates. One consensus phosphorylation site is p-Serine/Threonine-X-X-Serine/Threonine, where X refers to any amino acid[8,9]. This CKI consensus site requires priming by another kinase. CKI also phosphorylates an unprimed site, which optimally contains a cluster of acidic amino acids N-terminal to the target including an acidic residue at *n*-3 and a hydrophobic region C-terminal to the target[7,10]. A single acidic residue in the *n*-3 position is not sufficient for CKI phosphorylation. In contrast, in several important targets, such as β-catenin[11,12], CKI does not require *n*-3 priming, albeit less efficiently than the optimal sites[13].

In general, CKI kinases are constitutively active. The long C-terminal extensions of CKIδ and CKIε, however, are autophosphorylated, and this phosphorylation inhibits the activity of the kinase domain, although *in vivo* phosphatases keep it constitutively active in many cases[14]. Removal of inhibition by proteolytic cleavage or dephosphorylation of the tail is therefore required to fully activate these kinases[15]. Studies of regulation of CKIε and CKIδ in the canonical Wnt signaling pathway indicate that dephosphorylation, rather than proteolytic cleavage, is most likely the way *in vivo* for their activation[16,17]. This was evidenced by the fact that the activity of wild-type (WT) CKIε, but not of the constitutively active mutant form (MM2) in which all inhibitory phosphorylation sites are altered, was increased 5-fold upon Wnt ligand stimulation[16]. On the contrary, removal of the inhibitory tail actually impaired its proper function *in vivo* due to failure to form a secondary axis, even though its activity was intact *in vitro*[17]. Consistent with dephosphorylation as a preferential mode of activation *in vivo*, Takano *et al*[18] have identified a naturally occurring CKIε variant (S408N), in which one of the putative auto-inhibitory phosphorylation sites is mutated, leading to increased kinase activity. This was shown to have a protective effect against familial advanced sleep phase syndrome due to its ability to phosphorylate clock proteins, resulting in an elongated circadian rhythm[18].

Even though dephosphorylation has emerged as the predominant mode of activation for CKIε and CKIδ, structural analysis of CKIδ may have revealed an additional mechanism through which CKIε/δ could be regulated *in vivo* (Figure 2). Since Rivers *et al*[14] showed that, at the physiological level, constant phosphatase activity appears to keep CKIε and CKIδ in a hypo-phosphorylated state most of the time, which would presumably keep them active, this additional

mode of negative regulation could help minimize their activity even in a dephosphorylated state. Based on X-ray crystallography of CKIδ Δ317 and molecular replacement from the published crystal structure of the *Schizosaccharomyces pombe* homolog Cki1 (Cki1 Δ298)[19,20], Longenecker *et al*[19] compared the 3-dimentional (3D) structure of the core protein against its amino acid sequence. Through this more accurate comparison they were able to notice a broad surface across the catalytic domain, which is highly conserved between CKIε and CKIδ. Conservation in this region suggests a possible dimerization site, which could block their activations by physically covering the active site. Therefore, dimerization has also been proposed as an alternative mechanism of negative regulation for CKIε and CKIδ[4]. In support of this mechanism, preliminary structural analysis indicates that the CKIε point mutations previously identified in tumor tissue from six breast cancer patients with ductal carcinoma in situ (DCIS) are clustered within this putative dimerization domain (internal communication E. Brumovska and L. Trantirek)[21]. Moreover, based on their position within the 3D structure of the protein, it appears that accumulation of at least three of these point mutations in the same protein would be sufficient to disrupt dimerization (internal communication E. Brumovska and L. Trantirek). These findings could both explain why five of those six patients had multiple mutations in that region, and also suggest that inhibition of dimerization may contribute to tumorigenesis in patients with DCIS through increased overall kinase activity, providing support for dimerization as an additional mechanism of regulation *in vivo*.

GENERAL PATHOLOGY OF CKI

CKI members (α, γ, δ, and ε) have been implicated in several pathological conditions, including sleep disorders[18,22], Alzheimer's Disease (AD)[23] and cancers[4,21,24,25], particularly through identification of specific point mutations and/or changes in expression. Among them, CKIε and δ emerge as the most critical and positive players in these diseases. The recent findings on their active involvement in hyper-phosphorylation of tau protein and accumulation of toxic peptide Amyloid β, two primary characteristics of AD[26,27], further support this notion.

Similarly, the connection between CKIε/δ and cancers has been strengthened through identification of their targets in promotion of cell proliferation and/or inhibition of apoptosis, both of which can contribute to tumorigenesis. For instance, CKIε, δ as well as γ are found to be positive regulators of the canonical Wnt signaling pathway, which promotes cell proliferation through activation of proto-oncogenes like c-myc and cyclin D1, and which is also up-regulated in several malignancies, particularly colon cancer[4,28,29]. Moreover, our recent study showed that CKIε is a positive regulator of the Akt pathway[30], which is also activated in several cancers, including colon cancer[31] and breast cancer[32]. In addition, Akt signaling was also shown to contribute to resistance to a wide range of chemo-therapeutic drugs, making it a very attractive target for cancer treatment research[33-37]. Following up on the suggestion that CKIε may contribute to breast cancer based on both changes in protein expression and accumulation of CKIε point-mutations in tumor samples from DCIS patients[21], we found that CKIε up-regulates Akt in breast cancer cell lines in an independent manner of Phosphate and tensin homologue deleted on chromosome ten (PTEN)[30], the major inhibitor of the Akt pathway[38,39]. Moreover, inhibition with the CKIε/δ inhibitor IC261 was able to block phosphorylation of both Akt and its downstream target Glycogen Synthase Kinase 3β (GSK3β), suggesting that CKIε function is required for Akt activity[30]. A similar effect with IC261 was also seen in Hs578T breast cancer cells, in which the Akt pathway is normally up-regulated regardless PTEN, suggesting that, at least in breast cancer, CKIε/δ may contribute to a more tumorigenic environment through PTEN-independent Akt activation[30].

The connection between CKIε/δ and cancer has also been strengthened by their role in down-regulating apoptosis, particularly Fas-mediated apoptosis[4]. Stimulation by Fas ligand (CD95/APO-1) or agonistic antibodies leads to caspase 8 activation, which can either result in caspase-3 activation or in mitochondria-mediated cell death signaling through Bid cleavage by caspase-8. Desagher *et al*[40] showed that over-expression of CKIε stabilizes Bid, resulting in a lower number of apoptotic cells, while inhibition of CKI had the opposite effect. In addition, CKIε/δ have also been suggested to contribute to apoptosis by playing a role in the switch mechanism between canonical and non-canonical Wnt signaling, where they may promote canonical Wnt signaling at the expense of JNK-mediated apoptosis[41,42]. However, new evidence showing that CKIε and CKIδ can be activated not only by canonical Wnt3a[16] but also by non-canonical Wnt5a[43] has raised questions about the latter hypothesis.

CKI IN COLON CANCER

Last year, two independent studies provided strong evidence that CKIε/δ play a role in early stages of tumorigenesis predisposing to colon cancer. Umar *et al*[44] used the *Citrobacter rodentium*-induced transmissible murine colonic hyperplasia (TMCH) model, which allows studying changes during these early stages, and showed that CKIε protein levels as well as its activity are increased by 2- to 3-fold after 6 and 12 d of infection, suggesting an association between CKIε up-regulation and colon cancer development. Based on an increase in both Wnt target genes and S45-phosphorylated β-catenin, Umar *et al*[44] proposed that CKIε up-regulation contributes to colonic hyperplasia through activation of the Wnt pathway, as well as phosphorylation of β-catenin at S45. The notion that CKIε contributes to colon cancer through up-regulation of canonical Wnt signaling is not surprising, given that the pathway itself is activated in a majority of colon cancers, often through inducing mutations in key components

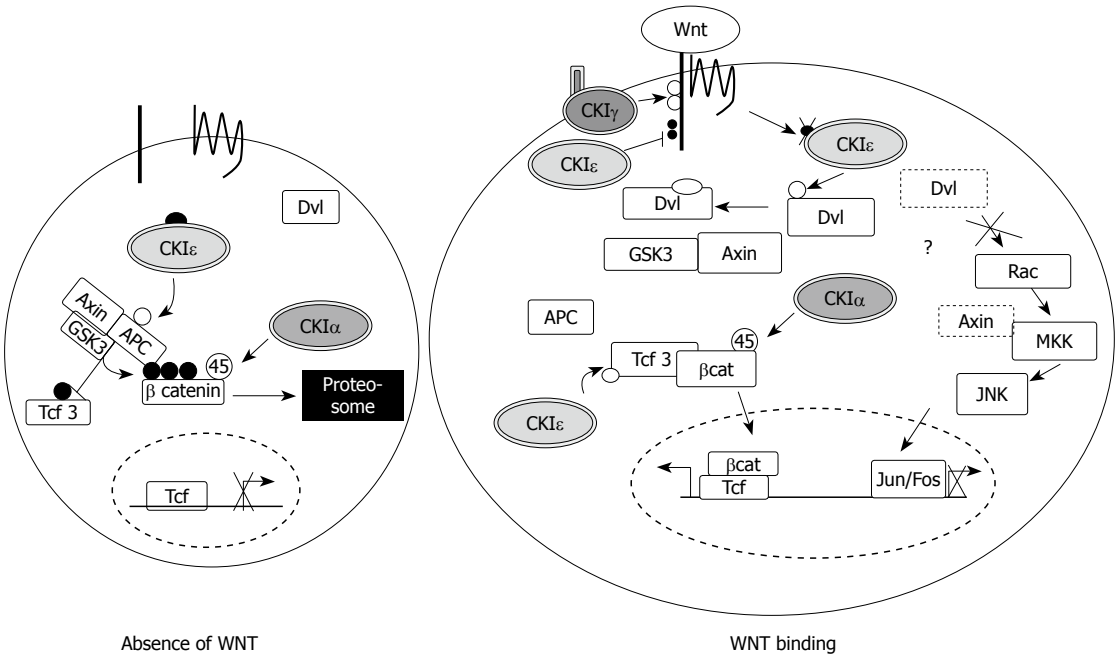

Figure 3 CKI members in canonical Wnt signaling. In the absence of Wnt ligand, β-catenin is phosphorylated by GSK3β after priming at S45 by CKIα and becomes degraded. In this state, CKIε phosphorylates APC and stabilizes the β-catenin degradation complex. Upon Wnt ligand binding to its receptor, CKIε is fully activated through dephosphorylation and contributes to the disassembly of the β-catenin degradation complex by phosphorylating Dishevelled (Dvl) and facilitating its interaction with Axin and GSK3β. Therefore, even though CKIα still primes β-catenin at S45, without additional phosphorylation by GSK3β, β-catenin becomes stabilized and accumulates in the cytoplasm. CKIε also promotes β-catenin stability by phosphorylating Tcf3 which competes against GSK3β for β-catenin. Stabilized β-catenin then translocates into the nucleus, where it contributes to activation of Wnt target genes. CKIε also negatively regulates the Wnt pathway by phosphorylating the LRP-co-receptor, which is positively phosphorylated by CKIγ. Since epistasis analysis showed that CKIε-mediated phosphorylation of LRP was downstream of its phosphorylation of Dvl, this is most likely a negative feedback loop to quickly deactivate the pathway after propagation of the signal.

such as Adenomatous polyposis coli (APC) and β-catenin[45]. However, their interpretation of increased β-catenin phosphorylation at S45 is questionable. First, the reported increase in β-catenin phosphorylation at S45 in TMCH is actually only subtle after normalizing to total β-catenin levels and therefore more likely due to "a proportional increase in overall β-catenin abundance"[44]. Moreover, even though two reports originally attributed priming phosphorylation of β-catenin at S45 to CKIε[11,46], Liu *et al*[12] convincingly showed that it is CKIα, not CKIε, that phosphorylates β-catenin at S45 *in vivo*. They further demonstrated that RNAi against CKIα rather than CKIε, inhibited S45 phosphorylation of β-catenin in 293T cells[12], making CKIα the most likely CKI member responsible for priming phosphorylation of β-catenin. In the absence of Wnt signaling, GSK3β can associate with the β-catenin degradation complex and further phosphorylate β-catenin. This additional phosphorylation targets β-catenin for degradation, therefore preventing it from activating Wnt-specific target genes. After Wnt stimulation, which results in full activation of CKIε[16], dephosphorylated CKIε disrupts the β-catenin degradation complex and prevents GSK3β from further phosphorylating β-catenin. Lack of additional GSK3β-mediated phosphorylation will result in stabilization and accumulation of β-catenin in the cytoplasm, which is required for its subsequent translocation to the nucleus to activate Wnt target genes (Figure 3). Therefore, the reported phosphorylation of β-catenin at S45 seen in TMCH is more consistent with CKIα phosphorylating β-catenin at S45, but still consistent with the authors' conclusions that CKIε up-regulation contributes to colonic hyperplasia through up-regulation of the Wnt pathway and subsequent increased transcription of Wnt target genes.

In a separate study based on a clinical family pedigree analysis of colon cancer[47], one patient was diagnosed at age 46 with several large polyps (5 mm or more), including one > 20 mm. This was approximately 10 years earlier compared to his siblings, suggesting a predisposition to early on-set of colon cancer. Screening identified a point mutation in a highly conserved region of CKIδ (R324H), which was correlated with the more severe condition of the patient through both *in vivo* phenotypic analysis and cell culture transformation potential[47]. This case further indicates that CKIε/δ contribute to early onset of colon cancer. More specifically, *in vivo* analysis of the R324H mutant in *Xenopus* revealed the axis duplication phenotype indicative of increased canonical Wnt signaling as well as an additional gastrulation phenotype that significantly contributed to the aggressiveness of CKIδ -related polyps[47]. Taken together, these results suggest that canonical Wnt signaling is not the only pathway that activates CKIε and CKIδ in colon cancer. The non-canonical Wnt/PCP and Wnt/Ca^{2+} pathways, which are involved in gastrulation, are also regulated by CKIε and δ[48-50]. Consequently, Tsai *et al*[47] tested known downstream components of these pathways, namely JNK, RhoA and NF-AT. However, they were not able to detect any changes in either of these pathways when using

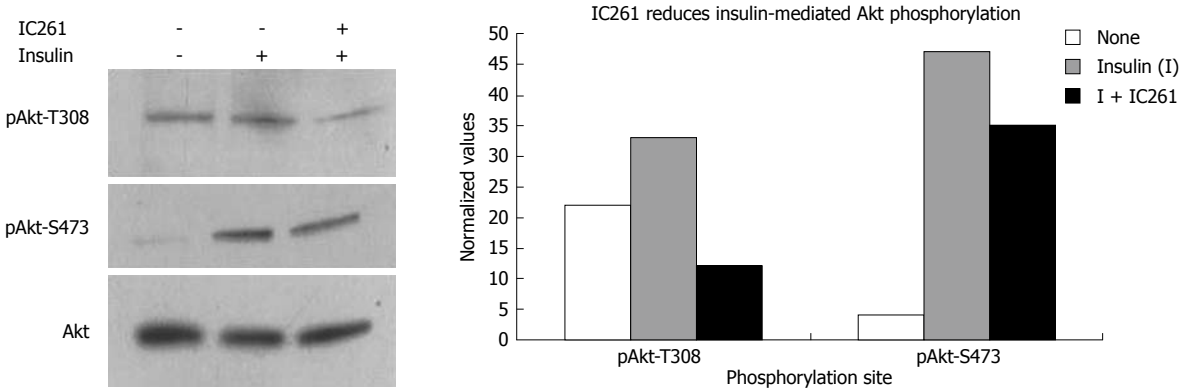

Figure 4 Pharmacological inhibition of CKIε/δ reduces insulin-induced Akt phosphorylation in PDAC cells. Cells were serum starved overnight and then incubated in 10 μg/mL insulin-containing media for 20 min in the presence or absence of the CKIε/δ inhibitor IC261 (40 μmol/L). Akt activation was measured by phosphorylation at T308 and S473. Bands were quantified using Image J software and values were normalized against total Akt.

the R324H mutant compared to WT, suggesting that the Wnt/PCP and Wnt/Ca^{2+} pathways are not likely affected by the CKIδ mutation.

CKI IN PANCREATIC CANCER

Earlier this year, CKIε and δ were also found in association with another gastrointestinal cancer, pancreatic ductal adenocarcinoma (PDAC), which is the most common type of pancreatic cancer[24]. Brockschmidt *et al*[24] showed that CKIε and δ are strongly expressed in both PDAC cell lines and actual tumors tissues. Importantly, inhibition of CKIε/δ by IC261 could effectively re-sensitize cells to apoptosis *in vitro* and reduce tumor growth *in vivo*. More specifically, prolonged treatment of low doses of IC261 in combination with the agonistic anti-Fas antibody CH11 increased cell death by 50% in pancreatic cancer Panc89 cells, which are normally resistant to Fas -mediated apoptosis. This was also correlated with increased Fas-mediated cleavage of caspase-8 and Bid, as well as activation of caspase-3 as measured by cleavage of its substrate PARP[24]. Moreover, IC261 treatment of SCID mice, which had been previously implanted with PancTu-1 cancer cells to develop pancreatic tumors, lead to a significant reduction in tumor size[24]. While IC261 was as effective as gemcitabine, a drug currently used to treat pancreatic cancer, combined treatment did not result in any additional benefit[24]. Real-time PCR analysis of tumor tissues treated with either drug revealed changes in expression of various genes[24]. Most notably, CKIε and CKIδ were decreased with both drugs, even though the change was greater with IC261 for CKIδ. Moreover, while most genes followed similar patterns between the two treatments, FASLG was strongly increased with IC261 while it was decreased with gemcitabine. Taken together, their data strongly suggests that CKIε/δ promote PDAC through their known anti-apoptotic role in Fas-mediated regulation. However, analysis of their tumor samples revealed that the reduction in tumor size was associated not only with an increase in apoptosis but also with a decrease in cell proliferation. This suggests that CKIε/δ contribute to tumorigenesis in PDAC also by promoting cell proliferation. Since we recently showed that CKIε can up-regulate the Akt[30], which is also up-regulated in PDAC[31,51,52], we tested whether IC261 can inhibit Akt signaling in PDAC cells as well. Our preliminary results show a decrease in Akt phosphorylation at both of its activating sites (Figure 4), indicating that CKIε-mediated Akt up-regulation is not only restricted to breast cancer. However, more studies would be required to determine how much the decrease in Akt signaling is contributing to the decrease in cell proliferation seen by Brockschmidt *et al*[24] in SCID mice treated with IC261.

CKI IN GASTRIC CANCER

In addition to their role in colon cancer and pancreatic cancer, preliminary data from different research labs strongly suggest that CKIε and CKIδ may also be involved in gastric cancer, the next most common gastrointestinal cancer in the United States after pancreatic cancer[5]. Recently, von Blume *et al*[53] showed that gastrin, a major stimulator of gastric acid secretion, increases CKIε/δ activity. This ultimately results in de-repression of HDAC7-regulated genes such as *nur77*. Nur77 has been shown as a potent oncogenic survival factor in several types of cancer, including lung, prostate, breast and colon cancer[54]. Therefore, while the focus of the study was more at the mechanistic level of signal transduction, these results indicate that CKIε/δ could play a role in gastric cancer as well. Further evidence in support of this comes from two separate studies on H-prune and its binding partner nm23-H1, which are key inducers of cell motility in breast cancer. Oue *et al*[55] recently showed that increased expression of H-prune and nm23-H1 was strongly correlated with tumor progression and poor survival in gastric cancer, while Garzia *et al*[56] provided mechanistic evidence on the interaction between H-prune and nm23-H1, implicating CKIε/δ as key mediators of this interaction. Out of the143 gastric cancer cases analyzed by Oue *et al*[55], 87% were positive for nm23-H1, which was expressed in 98% of H-prune positive gastric cancer cases. Many of the cases in which H-prune and nm23-H1 were co-expressed showed more advanced T

grade, N grade and tumor stage, and H-prune positive patients had significantly worse survival rate than H-prune negative patients, clearly supporting an important role for H-prune (and nm23-H1) in gastric cancer progression. Since co-expression of H-prune and nm23-H1 resulted in more aggressive tumor stages, the formation of H-prune/nm23-H1 complexes seems to play a critical role in their adverse effects, and disrupting those complexes could be potentially therapeutically advantageous. It is in this context that CKIε/δ could be again promising drug targets, since they phosphorylate a critical region within the H-prune binding region on nm23-H1 and their inhibition with IC261 (or competitive binding with phosphorylated nm23-H1 peptide) disrupted complex formation, resulting in inhibited cell motility[56].

CONCLUSIONS AND FUTURE DIRECTIONS

Results from recent studies in gastrointestinal cancers provide strong evidence of an association between CKI and carcinogenesis. However, they also raise important questions as to whether these kinases could be successfully targeted for cancer screening or treatment. For colon cancer, while activation of the canonical Wnt pathway was evidenced, results by Tsai *et al*[57] also indicate the possibility of some undefined pathway (related to cell migration and morphogenesis during gastrulation) that may be affected by CKI. Identifying this additional pathway is certainly a daunting task due to the complexity of gastrulation[58,59] and the wide number of known (and unknown) targets of CKI. However, at least two new targets of CKIε could be possible alternative candidates for the R324H mutant, given the great similarity between CKIε and δ. The first one is Rap1, an alternative GTPase that was later shown by the same group to promote gastrulation through CKIε[57,60]. The second is Akt, which we recently identified as a new target of CKIε[30]. Akt is also involved in cell migration, even though its role in gastrulation is still unclear[61]. Regardless which pathways are involved, it may be even more interesting to see whether the CKIε/δ-specific inhibitor IC261 could reduce hyperplasia and/or polyp formation effectively.

Several lines of evidence show that the CKIε/δ-specific inhibitor IC261 has potential therapeutic effects for cancer. On the one hand, CKIε/δ inhibition could be successfully achieved within non-toxic levels both *in vitro* and *in vivo*[24,27]. Moreover, IC261 was also shown to be more specific, resulting in fewer side-effects compared to some currently used alternatives[27,62,63]. For instance, while it has been shown to cause transient mitotic arrest, its effect is limited to mitosis, unlike other mitotic spindle drugs like nocodazole, which affect microtubule stability in general[62]. This feature could actually be advantageous to cancer therapy by targeting primarily actively dividing cancer cells, as was also shown *in vitro* by another group[63]. In addition, Behrend *et al*[62] also provided a more detailed analysis of the potential effects of IC261 in PDAC and showed that it is as effective as gemcitabine, the chemotherapeutic drug most commonly used for pancreatic cancer patients[27]. However, combinatorial treatment did not have any additive advantages[27], possibly because the two drugs for the most part affect the same pathways. This is tentatively supported by their gene expression data showing mostly similar trends in up and down-regulation of genes such as CKIε and CKIδ. These results highlight the importance of understanding at the molecular level which pathways contribute to the disease, how these pathways are regulated and connected, and which components are affected by specific drug targets. In this case, Brockschmidt *et al*[24] convincingly showed that IC261 promotes apoptosis in PDAC by blocking CKIε/δ-mediated inhibition of Fas-mediated signaling. However, their data also raises questions about which CKIε/δ-mediated cell proliferative signal is also inhibited by IC261. Answering this question would help determine what other drug could be more effective in combinatorial treatment. For instance, Morgan-Lappe *et al*[64] mentioned that the Akt specific inhibitor A443654 was successful in combination with specific inhibition by siRNA of the CKIγ3 isoform, which is another positive regulator of Wnt signaling[65]. However, this may not be the best candidate in combination with IC261, which is specific to only CKIε/δ, if IC261 is already blocking Akt signaling.

Taken together, results presented by Tsai, Umar, Brockschmidt, von Blume, Oue and Garzia further support a role of CKIε/δ in carcinogenesis, particularly of the gastrointestinal tract, and make them very promising markers for both early detection/risk assessment and cancer therapy. Understanding the mode of regulation of these kinases can provide insight into how naturally occurring CKIε/δ mutations work, and also help identify which portions of the gene are more likely mutated. This is particularly important for conditions like colon cancer, as well as many other cancers including pancreatic cancer, where asymptomatic early detection (i.e. through screening of mutations such as R324H) could be critical for better prognosis. More studies are required to further explore this area and also determine whether CKIε/δ can be pharmacological targets for treatment as well, particularly in light of the promising results with the CKIε/δ inhibitor IC261 against pancreatic cancer[24] and AD[27], both of which currently do not offer many successful options.

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GUIDELINES FOR CLINICAL PRACTICE

Positron emission tomography's changing significance in the treatment of esophageal cancer

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Abstract

Incidence of esophageal cancer has been rising, and Positron Emission Tomography (PET) is one tool that has shown utility and promise as a tool for staging, treatment response, and prognosis. PET delivery has evolved over time and is now frequently registered with a CT scan at the time of acquisition. However, resolution and confounders such as post-treatment radiation changes may limit clinical utility. PET has been shown to be helpful in staging, especially in evaluating for distant metastases. PET acquired after chemoradiation may give important prognostic information that can guide additional treatment decisions. Studies have had substantial variability in recommendations for the timing and manner of using PET for this purpose, and additional study is needed.

Key words: Positron emission tomography; Esophageal cancer; Staging

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INTRODUCTION

The incidence of esophageal cancer has been rising, with an estimated 146 726 new cases and 124 728 deaths worldwide[1]. The poor long term survival in patients with esophageal cancer is due partly to late detection of disease. Tumors often remain undetected until they are locally advanced or metastatic, leading to poor prognosis. Esophageal cancer staging is intended to group patients with similar prognosis for appropriate therapy. The accuracy of staging is contingent on the sensitivity and specificity of the tools available to the physician, as is ongoing management based on response to prior therapy. Positron Emission Tomography (PET) is one such tool that has increased in usage over the last several years. Investigators have sensed great promise with PET, and reports evaluating its utility have multiplied.

PET DELIVERY

PET is performed by injecting a patient with radio-labeled glucose [2-(fluorine 18) fluoro-2-deoxy-D-glucose] (FDG), which is concentrated in tissues with higher metabolic activity. Radio-labeled glucose is transported into active cells, phosphorylated, and then is unable to be metabolized further through glycolysis. The effect is concentration of decay emissions in metabolically active tissues, including cancer, as measured with a scanner.

PET is often obtained in conjunction with a CT scan using a dual gantry machine that can obtain both image

sets without moving the patient. Although this allows for a more accurate registration between the scans, the relative speed with which CT images are obtained contrasts with the relatively long period required for PET acquisition. This results in a disparity between the images. Normal organ motion within the body "smears" the PET image over time but does not have as much impact on CT scans. The variation between the CT and PET image is greatest at the diaphragm and can result in a 30%-50% change in the maximum uptake[2]. Regardless of this limitation, co-registered PET/CT is superior to PET and CT obtained separately or viewed side-by-side. Another limitation of all PET scans is the relatively low resolution inherent in the imaging process. The intensity of FDG uptake may be similar between tissue with few cells which are largely active and tissue with many cells which are moderately active in the same volume. This results in an inability to distinguish fine detail within the scan and therefore lower resolution.

The measured activity within a PET scan is calculated as the Standard Uptake Value (SUV) and is obtained by dividing the measured decay events in a given body volume by the expected decay events if the distribution of activity from the administered FDG were homogenous throughout (with attenuation and other corrections)[3]. PET scans reveal metabolically active tissue regardless of whether the activity is from malignancy, inflammation, or other causes. This, along with the limited spatial resolution mentioned previously, limits the interpretation of PET in oncologic management generally.

Regardless of PET's limitations, it has improved accuracy of staging and its value in post-therapy evaluation is recognized but not yet fully defined. There have been a number of recent studies suggesting new beneficial uses of the modality, but the findings have been somewhat mixed and are difficult to collectively summarize into a coherent, well-supported guideline.

PET holds particular appeal to oncologists because of its apparent complementarity to CT scans and other imaging obtained for staging. Staging scans have historically focused on the anatomy of the patient while PET allows for insight into the functionality of tissues by representing the metabolic activity of tumor and normal tissue. The combination of anatomic and physiologic information seems conceptually superior to anatomic information alone as it informs staging and therapeutic efforts. PET is now typically added to clinical assessment, diagnostic CT, endoscopic gastroduodenoscopy, and endoscopic ultrasonography for staging workup.

PET UTILITY IN STAGING

Patients with locally advanced disease are often treated with neoadjuvant chemoradiation followed by surgery. Several meta-analyses have shown a benefit in local recurrence, complete resection, and survival with trimodality therapy compared with surgery alone[4,5]. However, the addition of neoadjuvant therapy limits initial staging due to the absence of histopathological information. This raises the potential value of additional information that can be used for clinical staging such as through PET.

Esophageal cancer uses the AJCC TNM staging convention to represent primary, nodal, and metastatic disease respectively. The T stage depends on the invasiveness of the primary tumor and is well-appreciated with endoscopic ultrasound. PET scans may have value in determining the size and location of the primary malignancy, and thereby may be used to assist in radiation treatment planning target delineation, but these do not influence the T stage[6,7]. There are other limitations to PET in regard to primary tumor evaluation as well. Although most esophageal malignancies are hypermetabolic and manifest on PET, lesions less than 1 cm may be too small to be detected. Also, the spatial resolution of PET is inadequate to contribute to the T stage by suggesting a degree of invasion with any certainty even when it is positive.

PET may improve the accuracy of the N stage by distinguishing metabolically active lymph nodes from enlarged benign nodes. However, the low resolution of PET imaging makes it difficult to distinguish loco-regional lymph nodes from direct primary tumor extension, and metabolically active nodes may reflect sarcoidosis, granulomatous disease, reactive nodes, or other non-malignant conditions. Using PET for N staging also shares the T stage limitation of failing to identify microscopic disease or gross disease less than 1 cm.

The area in which PET has the greatest utility in esophageal cancer staging is in the assessment of distant metastases, the M stage. PET/CT may detect metastatic disease at unusual sites that may otherwise have been overlooked, and has thereby been shown to improve staging and prevent inappropriate surgery for patients with metastatic disease.

PET UTILITY IN TREATMENT RESPONSE

Patients with persistent disease after neoadjuvant therapy and prior to surgery have a poorer outcome and may best be managed without surgery[8,9]. A PET scan may be helpful in more accurately determining patient response to treatment to facilitate choosing appropriate additional therapy.

There have been mixed reports on this topic. A reduction in SUVmean or SUVmax between pre- and post-treatment PET scans was a predictor of pathologic response in some series, but the cutoff point varied widely between the studies (e.g. 10% to 80%) and typically has been chosen tailored to a retrospective data set rather than prospectively evaluated[8,10-14]. In other studies, persistent uptake within the primary tumor site on a single post-treatment PET correlated with residual viable tumor and poor survival[9,15-17]. However, the specific SUVmax value used in these series as a cutoff varied from 2.5 to 4.0, and unfortunately other recent studies similarly designed have concluded that a single post-therapy PET scan is not adequate in determining

response within the primary tumor[18-20].

There are several issues that may contribute to the disparate findings among these studies. Some studies examined only adenocarcinoma patient response while others were exclusively squamous cell carcinoma. Most were mixed. This may explain the relatively large difference in SUVmax cutoff values used to assess treatment response. Additionally, negative findings often remain unpublished and could be under-represented in the published literature. Retrospective studies are also widely understood to suffer from bias, and that seems particularly relevant in a group of studies with similar conclusions but widely disparate objective data.

Another possible reason for the range of findings in studies that address PET as a tool to assess clinical response is the changing technical format of PET administration. Earlier studies routinely obtained PET without CT using a separate transmission scan for attenuation correction. PET/CT uses CT data to perform attenuation correction and the difference in time acquisition results in mismatching. This may be corrected using respiration-averaged CT, but because independent PET was used for many of the earlier studies while PET/CT has been used most frequently recently may explain some of the disparity in findings. There are also disparities between treatment centers in FDG dose and attenuation correction procedures[2].

A potential limitation of post therapy PET is the esophagitis and ulceration that is induced by chemoradiation during treatment and which manifests as increased uptake on PET. Reactive uptake in non-malignant tissues increases three or more weeks after treatment, but tumor tissue uptake may not yet have diminished within the first week or two after treatment. The timing of PET is important to minimize the potential masking of high uptake in actual persistent disease[20,21].

PET has also been used as an assessment of treatment response after brief chemotherapy and prior to the full course of chemoradiation. This holds advantages for the group of patients who have a poor response to chemoradiation because surgical outcome is poorer after trimodality therapy than it would have been if surgery had not been delayed for neoadjuvant therapy. Lordick *et al*[22] reported in the Municon trial on the utility of PET when used as an earlier assessment of neoadjuvant treatment response. Patients were divided into responder and non-responder groups after administering two weeks of preliminary chemotherapy. Non-responders were allowed to proceed directly to surgery without additional neoadjuvant therapy while responders received the full course of chemoradiation. The results suggested the feasibility of a PET-guided treatment algorithm for esophageal cancer. Another study showed that PET/CT after two cycles of chemotherapy predicts pathologic response to neoadjuvant therapy and long-term outcome with a sensitivity of 93% and a specificity of 95%[10].

CONCLUSION

PET is useful in esophageal cancer for staging and evaluation of treatment response. However, this is only true when PET is carefully interpreted with awareness of its limitations. An awareness of the scientific basis for PET will allow physicians to interpret the results within the patient's overall clinical history, including timing of PET acquisition prior to biopsies and other procedures that confound results. Specific prognostic information and appropriate treatment management in response to PET evaluation will become better defined as additional studies, particularly prospective trials, are published in the future.

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Pancreatic cancer: A model cancer for the study of the therapeutic effects of anticoagulants

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Abstract

Cancer-related thromboembolic disease is a well recognized syndrome since first described by Armand Trousseau in 1865. Preventing the morbidity and mortality related to thromboembolism in these patients is becoming a priority research area with the advent of new anti-coagulants. It is only recently that randomized trials of improved quality are been undertaken to study this question. Many of these trials however are still not accounting for the heterogeneity of "cancer" in terms of anatomical site, histology, stage and treatment. This editorial review highlights why pancreatic cancer may serve as a model malignancy to study this question.

Key words: Pancreatic Cancer; Vascular; Thromboembolism; Vascular thromboembolic disease; Heparin; Anticoagulants

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INTRODUCTION

Pancreatic cancer has the worst prognosis of all adenocarcinomas in the human body. The incidence rates almost match the prevalence rates for this cancer reflecting the very low (2%-4%) five-year survivorship. In the United Kingdom in 2002, 7152 pancreatic cancers and 7040 deaths from pancreatic cancer were reported for a mortality rate of 98% while in the USA for the year 2006 an expected incidence of 33730 new pancreatic cancers will be accompanied by an expected 32300 pancreatic cancer related deaths giving a mortality rate of 96%. This cancer has become the 4th leading cause of cancer related death in the western world.

There are a number of particular problems that may contribute to the poor outcome of this cancer. These include a non-resectable primary cancer in 85%-90% of patients, resulting in a continuous risk of further local invasion and damage to vessels and other vital structures, the increased incidence of biliary stenting procedures with heightened risk of biliary sepsis and pancreatitis, and the frequent metabolic problems such as diabetes and malabsorption causing decreased resistance to infection and increased susceptibility to cardiovascular morbidity. A key additional contributor to morbidity and mortality in this group of patients is vascular thromboembolic disease (VTE)[1].

VTE AND PANCREATIC CANCER

It is quite sobering to note that a multitude of post-mortem and epidemiological studies have persistently highlighted pancreatic cancer as a malignancy with

Table 1 Impact of VTE on survival in the NCI-CTG PA.3 study

Variable	HR	95% CI	*P* value
Treatment effect			
Erlotinib	0.79	0.66-0.95	0.01
Placebo	1.0		
Thrombosis effect			
VTE	2.33	1.82-2.98	< 0.0001
No VTE	1.0		
Performance status			
ECOG 2+	1.69	1.35-2.12	< 0.0001
ECOG 0-1	1.0		
Extent of disease			
Local	0.60	0.48-0.74	< 0.0001
Distant	1.0		

Reprinted with permission© 2008 American Society of Clinical Oncology. All rights reserved. VTE: Vascular thromboembolic disease.

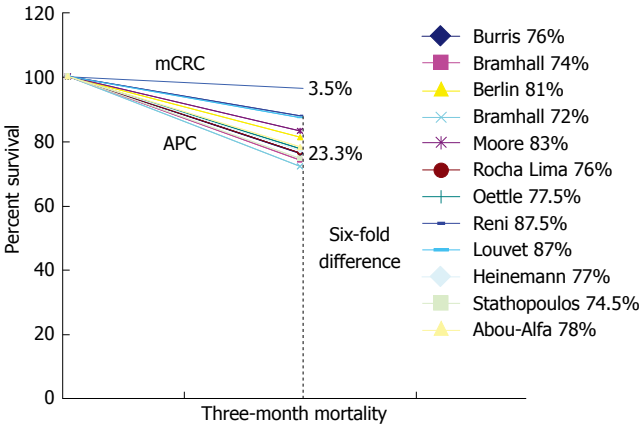

Figure 1 Early death burden (EDB) of patients with APC (advanced pancreatic cancer) compared to patients with mCRC (metastatic colorectal cancer). The key lists the trials reviewed and the figures (%) for 3-mo survival. Detailed data can be found in reference 7.

a severe propensity for thromboembolism[2-4]. The majority of patients will die with or of a VTE, and VTE is diagnosed premortem in 14 to 30% of patients and in up to 60% of patients at post-mortem[5]. Moreover at least epidemiological retrospective data have shown that the clinical occurrence of VTE confers a worse prognosis to patients with non-resectable pancreatic cancer[6]. The further tendency until recently for VTE not to be reported as an adverse event in registration chemotherapy trials has seemed to make the problem even more obscure[7]. The diligent reporting of VTE in the prospective sense in randomized trials can provide insight into the survival impact of VTE on pancreatic cancer as well. The additional data for the NCI-CTG PA.3 trial (Table 1)[8] demonstrate clearly that the detrimental impact of VTE on survivorship can be greater than the beneficial impact of the agent being studied (in this case erlotinib)[9]. We have shown that in fit patients randomized to phase Ⅲ trials the early portion of the survival curve is the steepest amongst all the cancers of the GI tract (Figure 1). We have suggested that this is an indirect effect of VTE related "stealth" mortality[7].

Before the advent of powerful and safe anticoagulants it would admittedly have been churlish to fault the lack of work in this area, given the risks and difficulties attached to warfarin and unfractionated heparin; however, today it is difficult to understand why the development of LMWHs in the last 15 years has not lead to the requirements of pancreatic cancer patients, vis a vis VTE, being marked as a priority area for research.

Could it be that to the casual eye reading the usual format of epidemiological data, pancreatic cancer does not seem to have such a seriously worse problem than other cancers? Why does pancreatic cancer tend to be grouped together with colorectal, ovarian and bladder cancer-amongst others- especially in the emerging major trials[10] that are now trying to redress the above iterated grievance?

Epidemiological registry studies seem to attribute a similar cumulative incidence of thromboembolism to the major cancers, such as ovarian cancer, colorectal cancer, stomache, lung and to pancreatic cancer. However these figures obscure a fact that does not seem to be usually highlighted. The common median survivorship of these different cancer groups is not taken into account. If one computes into the typically reported cumulative VTE incidence of 3%-5% for these cancers the fact that this incidence is seen over a median survivorship of 6-7 mo for metastatic pancreatic cancer, 20-24 mo for metastatic colorectal cancer and in excess of 36 mo for advanced ovarian cancer, one then gets a better feel for the disproportionate VTE related burden of APC patients throughout their short life-span. This has been best expressed in the article by Chew *et al*[4] where cumulative incidence was then computed in terms of rate of VTE per 100 patient-years. While the registry 2-year cumulative incidence of VTE for metastatic (the investigators use the term "remote") stomache, bladder, ovarian, colon and pancreatic cancer were 4.4%, 4.3%, 3.3%, 2.9%and 5.4% respectively, the rate of VTE per 100 patient-years for the same cancers for the first year after diagnosis were 10.7%, 7.9%, 3.6%, 4.3% and 20% respectively[4].

PANCREATIC CANCER: THE "MODEL" FOR THE STUDY OF VTE

There are at least 2 other malignancies in the metastatic setting with serious thrombosis related problems, gastroesophageal tract (GOC) and non small cell lung cancer (NSCLC). Interestingly, small cell lung cancer (SCLC) has a much diminished incidence of systemic VTE[11] but gets grouped with NSCLC as "lung cancer" in epidemiological studies reducing the actual VTE burden of "lung cancer". Similar to pancreatic cancer, patients with metastatic or locally advanced NSCLC and GOC tend to have short survivorship and high incidence of VTE. Although the thrombosis problem in these malignancies may not be as severe as in pancreatic cancer, they are more common malignancies and therefore a trial would potentially be easier to recruit. The reason however that makes pancreatic cancer a more compelling model for the testing of

anticoagulants is that it carries very few of the risks that would increase the likelihood of International Society of Thrombosis and Haemostasis (ISTH-scale) grade Ⅲ or worse bleeding complications. Brain metastases, with the accompanying increased risk of intracranial haemorrhage, while common in lung cancer are almost unheard of in pancreatic cancer. Nor is haemoptysis a risk in pancreatic cancer. Erosion of the GI tract and GI bleeding, although possible in some cases of duodenal infiltration, is markedly less likely than in GOC. Our own data attest to the safety profile of weight adjusted dalteparin in pancreatic cancer patients[12].

Why do we need a model for these studies? Recent meta analysis of trials to date have supported the notion that anticoagulants may have a significant impact on survivorship[13-15]. However, the inhomogeneous populations of cancer patients studied in terms of anatomy, histology, stage, performance status, concurrent treatments and "line" of treatment, coupled to the heterogeneity of the dosing schedules and the type of the anticoagulant used, mean that numerous confounding variables can be held up by the critics to account for the results. Standardization therefore through a "model" malignancy will be necessary to provide the type of data that will win over the skeptics. If cost-effectiveness of VTE prevention cannot be demonstrated in a cancer with such a high VTE-event-rate where maximum therapeutic impact can be achieved with minimum time on treatment, then success in other clinical settings would be uncertain.

Not realizing potential survival and quality of life benefits from the prevention of morbidity and mortality related to VTE may not be the only matter at stake. There may be further reasons why the study of anticoagulants specifically in pancreatic cancer should command attention. In an era of complex team approaches to trial design, often driven by statistical considerations and competing pharmaceutical priorities, clinical parameters that may influence trial endpoints may be easily overlooked. For example, the detrimental clinical impact of VTE on up to 20% of patients entering a pancreatic cancer study is an a priori variable that is not stratifiable. It therefore could influence the outcome of conventional study endpoints, putting major expensive trials of new anticancer agents at risk of both Type I and Type Ⅱ statistical error. This is especially pertinent in a cancer where we are looking for small benefits, where the accrual of more than a few hundred patients per trial is not likely and in which the superimposed thrombogenic effect of the anti-cancer agents (novel and old in combination) cannot be prospectively quantified. Maybe, in this specific malignancy, we should be fixing VTE first and then starting our journey down the exciting routes that seem to be opening up with the newer anti-cancer agents.

CONCLUSION

VTE in cancer is a complex and serious clinical issue that needs better understanding, better treatments and sophisticated preventative approaches. Pancreatic cancer patients are at the forefront of VTE related morbidity and mortality making pancreatic cancer the model malignancy to address the issues related to VTE and cancer.

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REVIEW

Hypermethylation of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia

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Abstract

A number of tumor suppressor and tumor-related genes exhibit promoter hypermethylation with resultant gene silencing in human cancers. The frequencies of methylation differ among genes and genomic regions within CpG islands in different tissue types. Hypermethylation initially occurs at the edge of CpG islands and spreads to the transcription start site before ultimately shutting down gene expression. When the degree of methylation was quantitatively evaluated in neoplastic and non-neoplastic gastric epithelia using DNA microarray analysis, high-level methylation around the transcription start site appeared to be a tumor-specific phenomenon, although multiple tumor suppressor genes became increasingly methylated with patient age in non-neoplastic gastric epithelia. Quantitative analysis of DNA methylation is a promising method for both cancer diagnosis and risk assessment.

Key words: Hypermethylation; DNA microarray; Tumor suppressor gene; Gastric cancer

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Tamura G. Hypermethylation of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia. *World J Gastrointest Oncol* 2009; 1(1): 41-46 Available from: URL: http://www.wjgnet.com/1948-5204/full/v1/i1/41.htm
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INTRODUCTION

DNA methylation is a type of epigenetic modification that introduces 5'-methylcytosine into DNA. It is estimated that approximate 70%-80% of CpG dinucleotides are heavily methylated in human cells[1]. However, CpG islands are protected from methylation, and approximately 60% of human genes are associated with one or more CpG islands[2]. Unmethylated CpG islands may become methylated in cancer cells with resultant loss of gene function[2]. Promoter hypermethylation prevents the binding of methylation-sensitive transcription factors and results in suppression of transcription[3,4]. During gastric carcinogenesis, promoter hypermethylation initially occurs in non-neoplastic gastric epithelia, increases with aging or following exposure to certain environmental factors, such as *Helicobacter pylori* infection, and ultimately silences gene function to constitute a field defect that may predispose tissues to the development of gastric cancer[5,6]. Many genes become methylated in gastric epithelia[6], although frequencies of methylation depend on which CpG sites within a gene promoter are examined[7]. Quantitative determination of hypermethylation in a particular genomic region in non-neoplastic gastric epithelia may be useful in gastric cancer risk assessment. In this review, the author describes: (1) age-related methylation of tumor suppressor and tumor-related genes, (2) how methylation spreads within promoter CpG islands, and (3) quantitative evaluation of methylation and its possible application for gastric cancer diagnosis and risk assessment.

Table 1 Methylation frequencies of tumor suppressor and tumor-related genes in non-neoplastic gastric epithelia from younger and eldery individuals

	< 32 years of age			> 44 years of age		
	U (n = 7)	M (n = 11)	L (n = 7)	U (n = 23)	M (n = 25)	L (n = 22)
APC	57	36	57	91	96	95
E-cadherin	0	0	0	78	76	64
DAP-kinase	0	0	0	78	80	68
p16	0	0	0	30	16	18
RUNX3	0	0	0	4	4	32
RASSF1A	0	0	0	4	12	5
hMLH1	0	0	0	4	0	14
GSTP1	0	0	0	0	0	0

U: Upper third portion; M: Middle third portion; L: Lower third portion.

AGE-RELATED METHYLATION OF TUMOR SUPPRESSOR AND TUMOR-RELATED GENES IN GASTRIC EPITHELIA

To clarify the physiological consequences of age-related methylation of tumor suppressor and tumor-related genes, the presence or absence of methylation was evaluated using methylation-specific PCR (MSP) in non-neoplastic gastric epithelia and other non-neoplastic cells of different tissue types obtained at autopsy. Results were compared between patients < 32 years old (n = 11) and patients ≥ 42 years old (n = 27)[6]. Results of this study demonstrated significant differences in susceptibility to age-related methylation among genes in different organs[6]. In non-neoplastic gastric epithelia, methylation was absent in younger individuals, except in promoter 1A of *APC* (Table 1). Methylation of this promoter is not oncogenic because another *APC* promoter (promoter 1B) is inherently protected from methylation; therefore, *APC* cannot be inactivated[8]. Hence, although present in younger individuals, *APC* methylation at promoter 1A does not contribute to gastric carcinogenesis. Methylation of other tumor suppressor and tumor-related genes was present at variable frequencies in non-neoplastic gastric epithelia from older individuals (Table 1). Methylation of *APC*, *E-cadherin*, and *DAP-kinase* was observed in the majority of samples; methylation of *p16* and *RUNX3* was found at intermediate frequencies; and methylation of *RASSF1A*, *hMLH1*, and *GSTP1* was rare or absent (Table 1). Thus, susceptibility to age-related methylation appears to significantly differ among various genes in gastric epithelia, although the frequency of methylation generally increases with age. Differences in methylation frequencies were also noted depending on the site in the stomach from which the sample was acquired. For example, *RUNX3* and *hMLH1* methylation was more frequent in the lower portion of the stomach (Table 1). The precise reasons for these phenomena are unclear. However, gastric cancer located in the antrum is known to be particularly susceptible to methylation of several tumor suppressor and tumor-related genes[9]. Intestinal metaplasia, particularly that of the incomplete type, commonly arises in the antrum and then expands toward the body of the stomach, and may therefore be predisposed to promoter methylation of these genes.

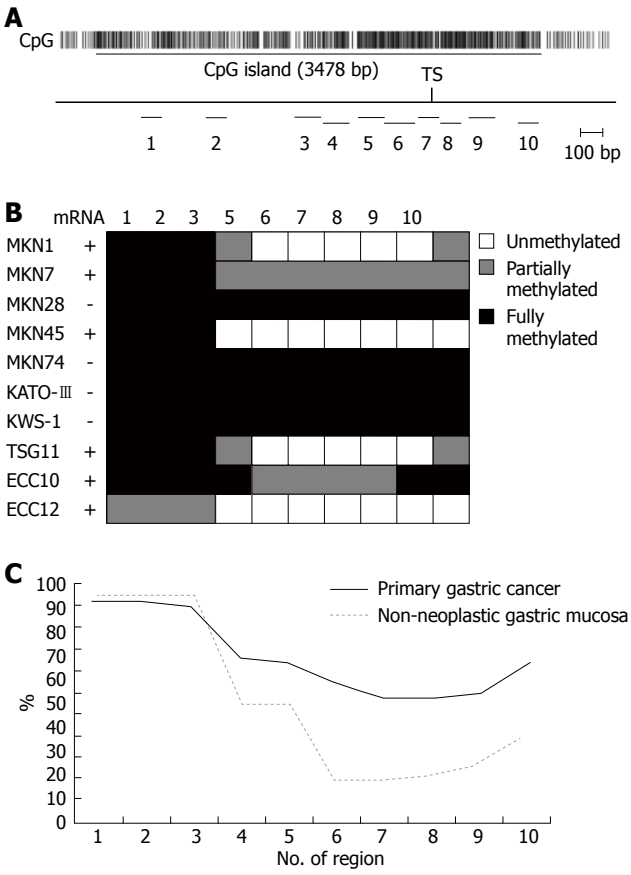

Figure 1 Methylation status of the *RUNX3* CpG island in primary gastric cancer and non-neoplastic gastric mucosa. A: *RUNX3* CpG island and analyzed regions (No. 1-10). CpG sites are shown as vertical bars. The transcription start site (TS) is located within region No. 7. B: Summary of methylation and expression status of *RUNX3* in gastric cancer cell lines. C: Percentage of methylation at multiple regions (No. 1-10) of the *RUNX3* CpG island in primary gastric cancer and non-neoplastic gastric mucosa.

SPREADING OF METHYLATION WITHIN PRMOTER CpG ISLANDS

Methylation and expression status of RUNX3 in gastric cancer cell lines

The methylation status of multiple regions within *RUNX3* CpG island was examined by MSP in 10 gastric cancer cell lines (MKN1, adenosquamous cell carcinoma; MKN7, well-differentiated adenocarcinoma; MKN28 and MKN74, moderately-differentiated adenocarcinomas; MKN45 and KWS-I, poorly-differentiated adenocarcinomas; KATO-Ⅲ, signet-ring cell carcinoma; TSG11, hepatoid carcinoma; and ECC10 and ECC12, endocrine cell carcinomas)[10]. Four (MKN28, MKN74, KATOⅢ, and KWS-1) of the ten gastric cancer cell lines were fully methylated at all the regions studied (Figure 1). These cell lines exhibited a loss of *RUNX3* mRNA expression that was restored following treatment with 5-aza-2'-deoxycytidine (5-aza-dc). The other six cell

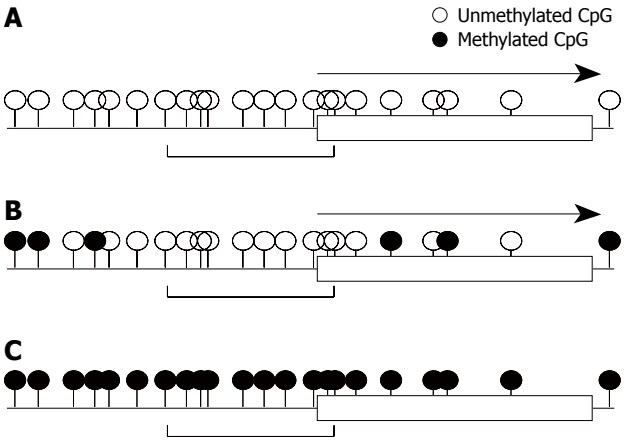

Figure 2 A schematic model of methylation spreading and gene silencing. A: Status of completely unmethylated CpGs with gene transcription (arrow); B: Appearance of methylated CpGs at the edge of CpG islands with gene transcription (arrow); C: Gene silencing by CpG methylation at the transcription start site.

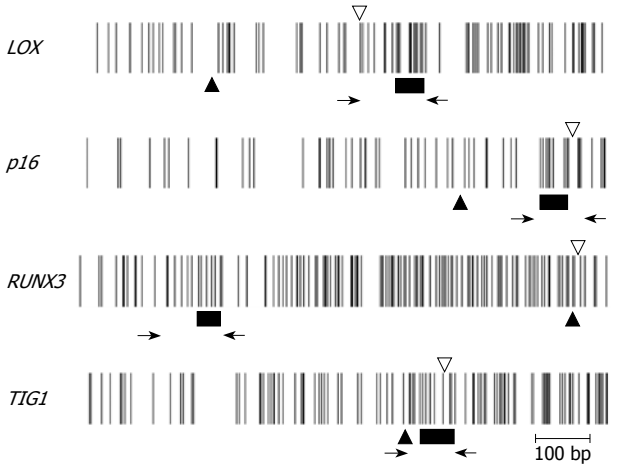

Figure 3 Locations of primers (arrows) and probes (boxes) used in microarray analysis, transcription start sites (closed triangles), and start codons (open triangles). Vertical bars indicate CpGs.

lines (MKN1, MKN7, MKN45, TSG11, ECC10, and ECC12) were either partially methylated or unmethylated at regions No. 5-8, which spanned the transcription start site, and expressed *RUNX3* mRNA (Figure 1). The 5' regions were generally more heavily methylated in all cell lines except for ECC12 (Figure 1). Thus, the critical region for *RUNX3* gene silencing evidently lies between regions No. 5-8, spanning the transcription start site[10].

Methylation status of RUNX3 in neoplastic and non-neoplastic gastric epithelia

The methylation status of *RUNX3* was compared between surgically resected gastric cancers and their corresponding non-neoplastic gastric epithelia[10]. Hypermethylation was detected at various regions in both neoplastic and corresponding non-neoplastic gastric epithelia (Figure 1). The methylation frequencies were very high at the 5' edge of the CpG island in both neoplastic and non-neoplastic gastric epithelia (Figure 1)[10]. In contrast, the methylation frequency near the transcription start site was very low in non-neoplastic gastric epithelia, although it remained high in neoplastic gastric epithelia (Figure 1)[10].

Spreading of methylation and gene silencing

We also studied mRNA expression and methylation status of multiple regions in the *RASSF2* CpG island in gastric cancer cell lines, and found that the edge of the CpG island was heavily methylated and that the critical region for gene silencing spans the transcription start site[11]. These results are very similar to the results for *RUNX3* described above. Furthermore, results of hypermethylation of multiple regions in the *RASSF2* CpG island in primary gastric cancers and corresponding non-neoplastic gastric epithelia were also similar to those of *RUNX3*[11]. Therefore, these may represent universal gene-silencing phenomena. Hypermethylation initially occurs at the edge of CpG islands and spreads to the transcription start site before ultimately shutting down gene expression (Figure 2).

QUANTITATIVE EVALUATION OF GENE METHYLATION

DNA microarray analysis

Multiplex PCR for *LOX*, *p16*, *RUNX3*, and *TIG1* was performed using primer pairs consisting of one Cy5-end-labeled primer and a phosphate-end-labeled reverse primer. Each primer pair was designed to simultaneously amplify both methylated and unmethylated DNA by precluding internal CpG sequences within the primers themselves (Figure 3). Control DNAs with varying methylation rates (0%, 25%, 50%, 75%, and 100%) were prepared by mixing CpGenome Universal Methylated DNA (completely methylated) and CpGenome Universal Unmethylated DNA (completely unmethylated). PCR products were hybridized to probes on a GenoPal™ microarray (Mitsubishi Rayon Co., Ltd, Tokyo, Japan) in a hybridization chamber (Mitsubishi Rayon Co., Ltd.). The microarray was scanned and the image was captured with a cooled CCD Microarray Image Analyzer (Mitsubishi Rayon Co., Ltd.) (Figure 4)[12]. Standardization curves were obtained based on the ratio of fluorescence intensity of the methylated sequence probe to the total fluorescence intensity of methylated and unmethylated sequence probes (Figure 4)[12].

Methylation rates in neoplastic and corresponding non-neoplastic gastric epithelia

Methylation rates (MRs) of *LOX*, *p16*, *RUNX3*, and *TIG1* in neoplastic and corresponding non-neoplastic gastric epithelia were measured and classified into five categories: level 1, 0%-20%; level 2, 20%-40%; level 3, 40%-65%; level 4, 60%-80%; and level 5, 80%-100%[13]. Level 5 methylation was not observed in any of the samples. MRs were generally low (< 10%) in non-neoplastic gastric epithelia, with level 2 methylation rarely observed. In contrast, levels 3 and 4 methylation appeared to be tumor-specific and were observed for *LOX* in 19% of samples, *p16* in 19% of samples,

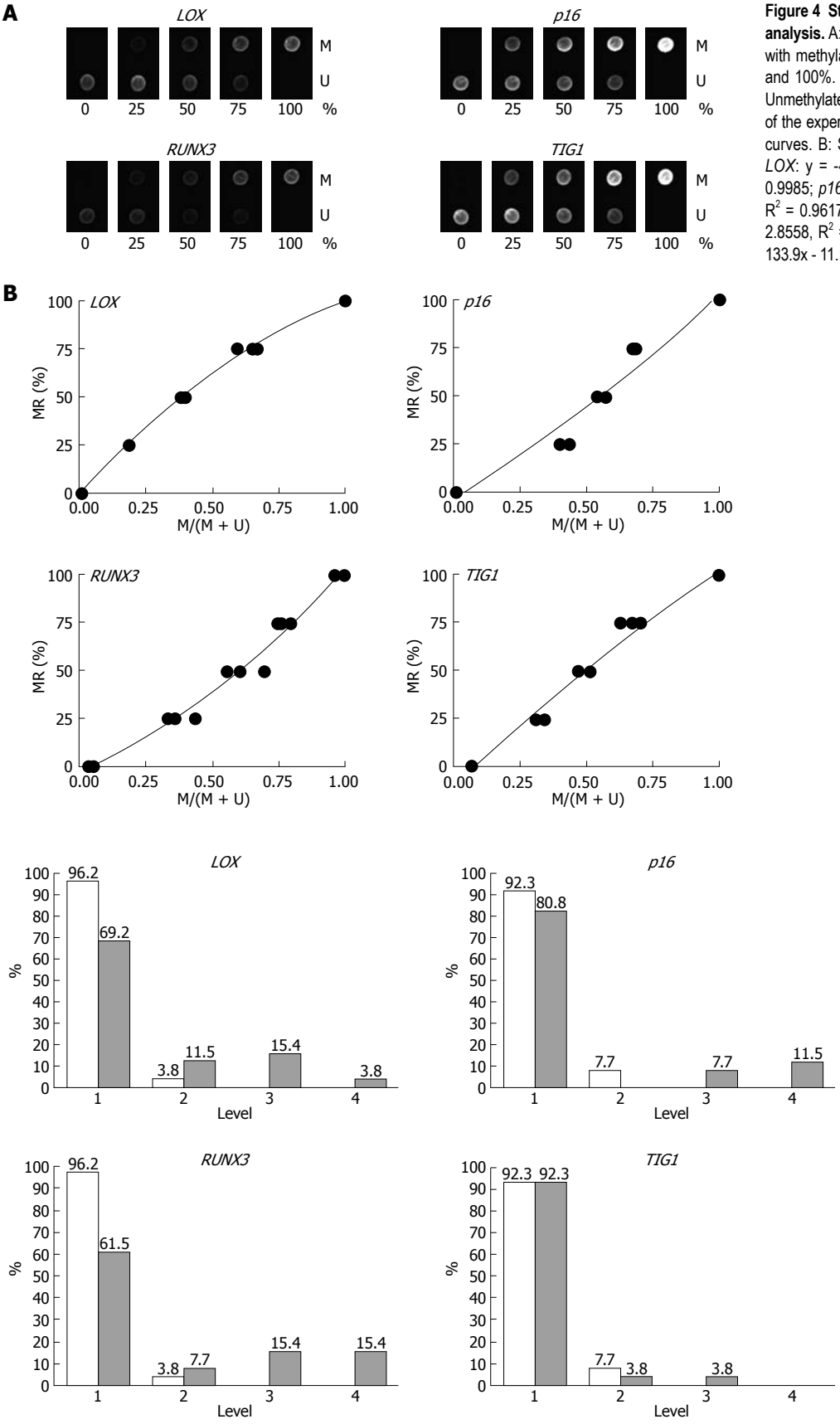

Figure 4 Standardization for DNA microarray analysis. A: Microarray images of control DNAs with methylation rates of 0%, 25%, 50%, 75%, and 100%. M: Methylated sequence probe; U: Unmethylated sequence probe. Three replicates of the experiment were used to create standard curves. B: Standard curves were obtained for *LOX*: $y = -49.256x^2 + 150.4x - 1.0847$, $R^2 = 0.9985$; *p16*: $y = 24.965x^2 + 81.528x - 2.9098$, $R^2 = 0.9617$; *RUNX3*: $y = 44.245x^2 + 61.71x - 2.8558$, $R^2 = 0.984$; and *TIG1*: $y = -20.932x^2 + 133.9x - 11.113$, $R^2 = 0.9852$.

Figure 5 Methylation rates of tumor suppressor genes in primary gastric cancers (gray bar) and non-neoplastic gastric epithelia (white bar).

RUNX3 in 38% of samples, and *TIG1* in 4% of samples (Figure 5). Thus, a high level of methylation, which indicates clonal expansion of methylated cells, appears to be a tumor-specific phenomenon[13].

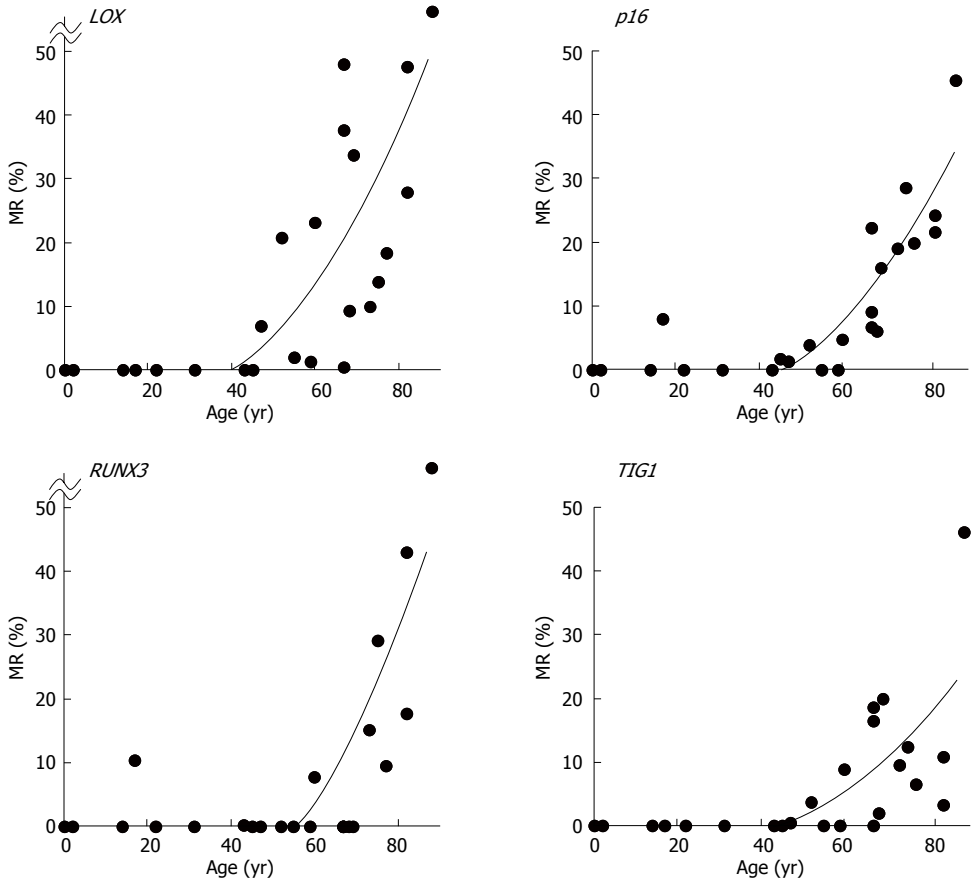

Figure 6 Regression curves. *LOX*: $y = 0.013x^2 - 0.6184x + 4.0512$, $R^2 = 0.5728$, $P < 0.001$; *p16*: $y = 0.0107x^2 - 0.6055x + 5.2943$, $R^2 = 0.7891$, $P < 0.00001$; *RUNX3*: $y = 0.0182x^2 - 1.2234x + 11.566$, $R^2 = 0.5595$, $P < 0.001$; and *TIG1*: $y = 0.0068x^2 - 0.3586x + 2.4306$, $R^2 = 0.4670$, $P < 0.01$.

Methylation rates in non-neoplastic gastric epithelia of patients without gastric cancer

MRs ranged from 0.0% to 77.2% (mean, 15.8%) for *LOX*, 0.0% to 45.8% (mean, 10.0%) for *p16*, 0.0% to 83.8% (mean, 9.0%) for *RUNX3*, and 0.0% to 46.1% (mean, 6.6%) for *TIG1* in non-neoplastic gastric epithelia of patients without gastric cancer[12]. A general increase in methylation of multiple tumor suppressor genes was observed beginning at 50 to 60 years of age, and the MR of each gene positively correlated with increased age ($P < 0.01$ by Spearman's rank correlation test (Figure 6)[12]. These data strongly correlate with the finding that gastric cancer incidence and mortality increase with age, particularly after age 50-60[14]. Thus, these results are consistent with the notion that age-related methylation is associated with cancer susceptibility in older individuals.

PERSPECTIVES

The construction and use of DNA microarrays specific for individual cancer types could ultimately be used to facilitate both cancer diagnosis and risk assessment. An age-matched control study of MRs in non-neoplastic cells or tissues of cancer patients and individuals without cancer should set a new standard for cancer diagnosis and risk assessment.

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REVIEW

Newly emerging standard chemotherapies for gastric cancer and clinical potential in elderly patients

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Abstract

With the increase in average life expectancy, the rate of occurrence of gastric cancer in elderly patients is also rising. While many clinical trials have been conducted to examine the effect of chemotherapy treatment on gastric cancer, age limits for eligible subjects have prevented the establishment of standards for chemotherapy in elderly patients with gastric cancer. As of March 2009, evidence-based standard chemotherapy regimens were established. In the Western world, debates centered on the ECF (Epirubicin/cisplatin/5-FU) or DCF (Docetaxel/cisplatin/5-FU) regimens based on the phase Ⅲ randomized controlled trial at the Royal Marsden Hospital (RMH) or the V325 study, respectively. The JCOG9912 and SPIRITS trials emerged from Japan indicating attractive regimens that include S-1 for advanced gastric cancer patients. Using these active anticancer drugs, the trials that studied the efficacy of adjuvant therapies or surgical approaches, such as the Int-116/MAGIC/ACTS-GC trials, have actually succeeded in demonstrating the benefits of adjuvant therapies in gastric cancer patients. For cases of gastric cancer in elderly patients, treatment policies should consider these studies while analyzing not only the therapeutic effects but also drug toxicity, individual general health conditions, and social factors to select treatments that emphasize quality of life.

Key words: Gastric cancer; Elderly patients; Chemotherapy; Regimen comparison

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INTRODUCTION

In 2002, there were an estimated 934 000 new cases of gastric cancer world-wide, accounting for 8.6% of all carcinomas, making gastric cancer the fourth most common cancer following lung cancer, breast cancer, and colorectal cancer. In addition, the mortality rate for gastric cancer is only second to that of lung cancer, with approximately 700 000 fatal cases, indicating a poor prognosis compared to breast cancer or colorectal cancer. With decreases in salt intake due to progress in methods of storing and preserving food, the incidence of gastric cancer is decreasing in many advanced countries, however, due to increases in population and average age, the number of afflicted patients in 2010 is predicted to be approximately 1 100 000 worldwide. Areas with a high incidence rate of gastric cancer include East Asia, Eastern Europe, and South America, while the incidence of gastric cancer is low in Africa, South

Asia, North America, Australia and New Zealand[1].

Japan has the highest incidence rate of gastric cancer in the world, despite a decrease since 1990, and gastric cancer remains the most common form of cancer in relation to site of organ[2]. Patients 65 years or older account for two-thirds of all cases, while patients 70 years or older constitute half of all cases; thus the treatment of gastric cancer in elderly patients has become a particularly important issue in countries with a large elderly population.

Regarding chemotherapy for gastric cancer, many clinical trials have been conducted to establish standard treatments. However, many of these clinical trials impose age limits for eligible subjects, targeting subjects up to 75 years of age in most cases, and cases with impaired organ function, which are common among elderly patients, are excluded. Therefore, it is difficult to evaluate the efficacy and safety of chemotherapy treatment for gastric cancer in elderly patients using only the results from these clinical trials. In this manuscript, we first review standard evidence-based chemotherapy for gastric cancer in both the West and Japan, and then we discuss the clinical potential of therapeutic applications and future prospects for elderly gastric cancer patients.

APPROACHES TO CHEMOTHERAPY FOR ELDERLY PATIENTS

It must first be noted that in chemotherapy for elderly patients functional impairments of the liver, kidneys, and lungs, which provide the main metabolic and excretory routes for drugs, can cause increases in blood concentrations that easily result in adverse reactions. Key organ functions deteriorate with age, but the level of deterioration varies greatly among individuals and the indication of chemotherapy cannot be determined on the basis of chronological age alone. If a patient's performance status is good and their key organ functions are maintained and there are no uncontrollable complications, it is considered possible to perform standard chemotherapy even with elderly patients. However, in some cases, unexpected adverse events occur in elderly patients due to functional deterioration that was not apparent from laboratory test values. Thus, a thorough understanding of adverse events that can be caused by anticancer drugs, together with careful observation and quick responses to adverse reactions, is required in elderly patients.

CHEMOTHERAPY FOR INOPERABLE OR RECURRENT GASTRIC CANCER

In a randomized comparative study of non-resectable or recurrent (far advanced) gastric cancer subjects, which were not thus indicative of surgical treatment, with a performance status (PS) of 0-2 in a symptomatic therapy (best supportive care, BSC) group not receiving anticancer drugs and a chemotherapy group receiving anticancer drugs, survival time was extended in the chemotherapy group, thus confirming the usefulness of chemotherapy in gastric cancer[3-5]. It has therefore been accepted that chemotherapy should be the first option for cases of far advanced gastric cancer with relatively good general health conditions.

The response rate for single agents that have been conventionally used for far advanced gastric cancer, such as 5-fluorouracil (5-FU), cisplatin (CDDP), adriamycin (ADR), epirubicin (EPI), and mitomycin C (MMC), is generally approximately 10% to 20%, and studies of combination therapies have been conducted to obtain higher therapeutic effects[6]. On the other hand, regimens such as 5-FU + ADR + MMC therapy (FAM therapy), 5-FU + ADR + methotrexate therapy (FAMTX therapy), 5-FU + CDDP therapy (CF therapy), 5-FU + leucovorin therapy (FL therapy), EPI + CDDP + 5-FU (ECF therapy), and irinotecan + CDDP therapy have obtained higher response rates of approximately 30% to 50% in phase Ⅱ studies. Life prolongation and/or quality of life (QOL), however, do not necessarily correlate with these response rates. Any such correlation must be ultimately verified in a phase Ⅲ comparative study for promising regimens using survival time and QOL as indices.

The results of one milestone comparative study conducted by the European Organization for Research and Treatment of Cancer (EORTC) indicated that FAMTX therapy is superior to FAM therapy[7], but in a subsequent randomized controlled trial (RCT) at the Royal Marsden Hospital (RMH), ECF therapy was shown to be further superior to FAMTX therapy in both the response rate and survival time (Figure 1A)[8]. In the ECF regimen, EPI was chosen instead of ADR because of its lower toxicity. Grade 2 alopecia representing pronounced or total reversible hair loss is characterized by Anthracyclins (EPI or ADR)-including regimens (Table 1). Based on these results, in Europe, ECF therapy has been considered the standard treatment. Recently, however, in a two by two design, a comparative study of EPI-included therapy (Real-2) was conducted, using combinations such as EPI + CDDP + capecitabine or 5-FU therapy (ECX or ECF therapy), and EPI + oxaliplatin + capecitabine therapy or 5-FU (EOX or EOF therapy), and the best combination was shown to be capecitabine and oxaliplatin with EPI (EOX therapy), which overcame ECF therapy with a hazard ratio for death of 0.80 (95% CI, 0.66-0.97, $P = 0.02$)[9] (Figure 1A).

On the other hand, in the V325 study in the United States, docetaxel + CDDP + 5-FU therapy (DCF therapy) was shown to produce significantly improved results compared to CF therapy in both response rate and survival time[10], and it is now considered to be one of the standard treatments (Figure 1B). This therapy has a significantly increased toxicity, however, exceeding grade 3 (neutropenia of 82%, diarrhea of 21%, nausea/emesis of 19%, anemia of 18%, lethargy of up to 19%, and thrombocytopenia of 8%, as shown in Table 1).

Using the results of a meta-analysis, Wagner *et al*[11] reported that combination therapy is more effective than single-agent therapy, and that therapy with a three-drug regimen is more effective than therapy with a two-

Table 1 Grade 3/4 side effects in active regimens for advanced gastric cancer

	FAMTX	ECF	EOX	CF	DCF	S1	CS
Anemia	10	8.0-13.1	8.6	26	18	4.0-12.8	26
Thrombocytopenia	8	4.0-4.7	5.2	13	8	0.0-1.3	5
Neutropenia	58	36.0-41.7	27.6	57	82	5.6-11.0	40
Febrile neutropenia	20	8.0-9.3	7.8	12	29	0.0-1.0	3
Diarrhea	7	2.6-6.0	11.9	8	19	3.0-7.7	4
Stomatitis		1.3	2.2	27	21	0.0	1
Hand-foot syndrome	1	3.0-4.3	3.1			0.0	0
Nausea and vomitting	5	10.2-17.0	11.4	17	14	1.0-5.6	11 and 4
Peripheral neuropathy		0.4	4.4	3	8	2.0	0
Lethargy		16.6	24.9	14	19	2.0-5.1	4
Alopecia[1]	42	44.2-56.0	28.8				
Increased creatinine	3	1.0				0.0	0
Ref.	[8]	[8,9]	[9]	[12]	[12]	[16,17]	[16]

[1]Alopecia is grade 2 according to CTCAE ver2. FAMTX: 5-FU/Adriamycin/Methotrexate; ECF: Epirubicin/cisplatin/5-FU; EOX: Epirubicin/Oxaliplatin/Capecitabine; CF: Cisplatin/5-FU; DCF: Docetaxel/cisplatin/5-FU; CS: S-1/Cisplatin.

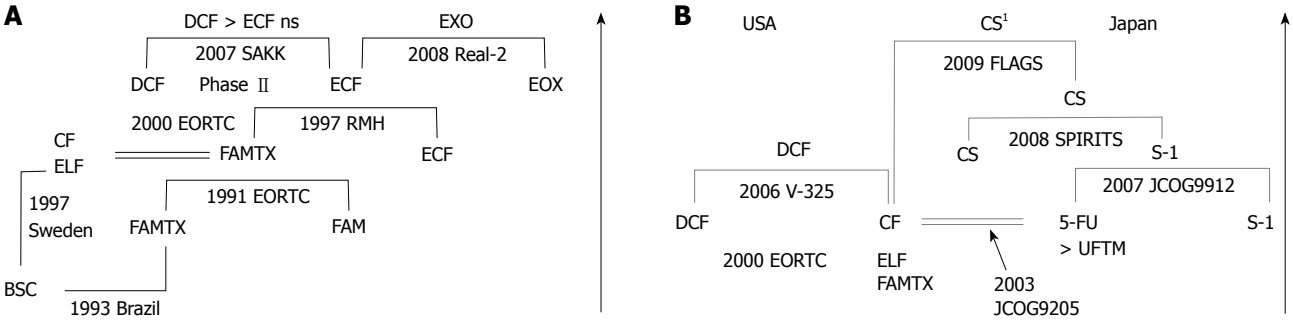

Figure 1 Phase Ⅲ trials are the most successful for far advanced gastric cancer in Europe (A) and in both United States and Japan (B). Arrows show chronological direction. BSC: Best supportive care; FAMTX: 5-FU/Adriamycin/Methotrexate; CF: Cisplatin/5-FU; ELF: Etoposide/Leucovorin/5-FU; FAM: 5-FU/Adriamycin/Mitomycin-C; ECF: Epirubicin/Cisplatin/5-FU; EOX: Epirubicin/Oxaliplatin/Capecitabine; DCF: Docetaxel/Cisplatin/5-FU; EORTC: European Organization for Research and Treatment of Cancer; RMH: Royal Marsden Hospital; SAKK: Swiss Group for Clinical Cancer Research; ns: Not significant. UFTM: UFT/Mitomycin-C; CS: S-1/Cisplatin; [1]CS regimens in the FLAGS study used 25 mg/m^2 of S-1 differently from the SPILITS trial (40 mg/m^2 of S-1).

drug regimen. In addition, the Swiss Group for Cancer Research conducted a phase Ⅱ trial to compare ECF therapy, docetaxel + CDDP therapy (DC therapy), and DCF therapy. They reported that the response rate was the best for DCF therapy, though there were no differences in survival times, putatively due to a small number tested in the phase Ⅱ trial[12] (Figure 1A). From the results of these clinical trials, in Europe and the United States, combination therapy using two or three drugs is considered standard treatment for cases with relatively good general health, but neither the Wagner *et al*[11] nor the Swiss group study discussed therapeutic effects or safety for patients in different age groups, and thus the best Western regimens for elderly patients remain elusive.

Trumper *et al*[13] analyzed the relationship between age (70 years older and under 70 years old) and therapeutic effects and safety in 1080 cases that had been registered in 3 studies conducted in the United Kingdom. Although some treatment methods included only a small number of subject cases aged 70 years or older, suggesting caution in evaluation, in all treatment methods [including regimens of CDDP (platinum-containing regimen), ECF therapy, 5-FU continuous intravenous infusion (PVI 5-FU) ± MMC therapy, and FAMTX therapy], there were no differences in either the therapeutic effects (response rate and survival time) or toxicity between either age group, and treatments were considered to be useful even for those aged 70 years or older. From this data, age may not be a serious issue to consider in administrating chemotherapy.

In Japan, independent clinical trials have studied treatment methods for far advanced gastric cancer (Figure 1B). In the JCOG 9205 study conducted in the 1990s[14], tegafur-uracil (UFT) + MMC (MC) therapy and CF therapy were evaluated and compared to monotherapy with 5-day continuous intravenous infusion of 5-FU (5-FU ci). There were no significant differences in survival time, which was the primary endpoint, although CF therapy yielded a superior response rate. Therefore, in Japan, 5-FU ci has been considered to be a standard therapy until recently.

S-1, an oral fluorinated pyrimidine drug developed in Japan in the 1990s, has obtained response rates exceeding 40% when used as a single agent, which is comparable to the response rates for conventional combination therapy[15,16], and there were high expectations that it would be a useful treatment method for advanced and recurrent gastric cancer. In the JCOG 9912 study, the noninferiority of single S-1 therapy compared to 5-FU ci and the superiority of irinotecan + CDDP therapy were

examined[17]. For overall survival, all other treatment methods yielded better results than 5-FU ci, where noninferiority of single S-1 therapy against 5-FU ci was confirmed statistically, but the superiority of irinotecan + CDDP therapy against 5-FU ci was not demonstrated. Concerning safety, irinotecan + CDDP therapy lead to leukopenia, neutropenia, and loss of appetite exceeding grade 3 at a rate exceeding 30%, while single S-1 monotherapy had a toxicity equivalent to that of single 5-FU therapy and a low incidence rate of adverse events exceeding grade 3 (Table 1)[17].

In the SPIRITS trial, conducted at approximately the same time as the JCOG 9912 study, S-1 + CDDP (CS) therapy showed significantly better results for overall survival and progression-free survival than S-1 monotherapy[18], where the incidence rate of leukopenia (11%), neutropenia (40%), anemia (26%), nausea (11%) and loss of appetite (30%) exceeding grade 3 was higher in CS therapy than in single S-1 monotherapy. Based on the results of these studies, in Japan, CS therapy is currently considered a standard therapy for far advanced gastric cancer.

Subsequently, a comparative study of S-1 monotherapy and S-1 + irinotecan therapy failed to show a significant difference in overall survival[19] and, currently, in a joint study in Japan and South Korea, a comparative study of single S-1 therapy and S-1 + docetaxel therapy is being conducted (Clinical Trials.gov Identifier: NCT00287768).

Regarding the toxicity of S-1 (adverse events exceeding grade 3), the results of a post-marketing survey of 3808 cases showed no differences with the initial survey in the incidence rate of adverse events, which suggests S-1 could be a safe agent for advanced gastric cancer. However, the incidence rate of adverse events is high in cases with reduced creatinine clearance, thus indicating the need for caution when administering drugs in cases with impaired renal function, such as in elderly patients[20].

In Japan, treatments using S-1 are taking a leading role, and when S-1 is combined with CDDP, the median overall survival (MST) exceeds 1 year. Even with S-1 alone, an MST of 11 mo has been obtained. Although a simple comparison is not possible, this exceeds the MST of the three-drug combination therapies used in the Western world. In addition, while dosages of S-1 need to be reduced due to the effects of racial differences in CYP2A6 gene polymorphisms in Europe and the United States[21], a Phase Ⅰ/Ⅱ study has obtained an MST of 10.4 mo, and a global Phase Ⅲ study (FLAGS study) comparing CF therapy with CS therapy revealed that S-1 did not show a significant benefit for survival compared to 5-FU in combination treatments of CDDP, however, many side effect was clearly reduced[22]. Its use is likely considered to be one of the most promising standard treatments around the world.

The proportion of elderly patients in recent phase Ⅲ clinical trials on far advanced gastric cancer worldwide is shown in Table 2. Only the SPIRITS trial disclosed the actual proportion of elderly patients 70 years old or over (17%) and, interestingly, recent studies have tended to include more elderly patients. These findings may suggest that chemotherapy has been judged as feasible even for elderly patients. It is important to expand the number of treatment options for elderly patients and to provide effective and less toxic treatment. In the SPIRITS trial, the therapeutic effects of CS therapy were better than single S-1 therapy in the subgroup of subjects aged under 60 years (hazard ratio, 0.75; 95% CI, 0.61-0.92), but no difference in effects were seen in the subgroups aged 60-69 years and subjects aged 70 years or older[18]. On the other hand, the most recent study of Arbeitsgemeinschaft Internistische Onkologie (AIO) revealed that oxaliplatin (FLO therapy) in replacement with CDDP (FLP therapy) was significantly effective only in patients older than 65 years, suggesting that oxaliplatin seemed to be very promising in elderly patients with far advanced gastric cancer[18,23].

ADJUVANT CHEMOTHERAPY FOR ADVANCED GASTRIC CANCER

When evaluating the indications for postsurgical adjuvant chemotherapy in gastric cancer cases, the standard options for resection, upon which the chemotherapy is based, are important. One problem is the range of lymph node dissection in Europe and the United States is different from that in East Asian countries, such as Japan. While D2 surgery is a standard procedure in East Asia, D2 surgery is not performed as standard in Europe or the United States. However, even in Japan where D2 surgery is performed as a standard procedure with curative intent, the 5-year survival rate is 69% in Stage Ⅱ, 50% in Stage ⅢA, and 28% in Stage ⅢB[24], which are not satisfactory outcomes for curative gastric cancer. Surgery alone does not improve survival rates, and therefore it is believed that some level of adjuvant therapy is required. There are many reports from around the world regarding postoperative adjuvant chemotherapy for gastric cancer in which meta-analysis has indicated extended survival times[25-30], but none of these evaluations used a particular regimen, and it had been concluded that examinations based on large-scale comparative studies are required.

In an INT-116 study conducted in the United States, postoperative adjuvant radiation chemotherapy (5-FU + leucovorin + radiation) lead to significant improvements in overall survival time and recurrence-free survival time and significant reductions in the local recurrence rate, compared with cases undergoing surgery only[31]. On the other hand, in a Magic trial conducted mainly in the United Kingdom, preoperative and postoperative chemotherapy (perioperative chemotherapy) with ECF therapy, which is even effective for far advanced gastric cancer, significantly extended the overall survival time and recurrence-free survival time[32]. Based on these results, postoperative radiation chemotherapy or perioperative chemotherapy with ECF therapy is a standard adjuvant therapy in the Western world.

Table 2 Phase Ⅲ milestone trials of chemotherapy for far advanced gastric cancer and information of the elderly patients

Publication	Trial	Regimens	OS (number)	Selected regimens	Median	Age range	Proportion of elderly patients (70 years old or over)	Ref.	Country
1991	EORTC	5-FU/Adriamycin/MMC (FAM)	4 (79)	FAMTX	58	23-69	No information	[7]	EORTC
		5-FU/Adriamycin/Methotrexate (FAMTX)	6 (81)		57	28-77			
1993	Murad *et al*	FAMTX	9 (30)	FAMTX	58	26-72	No information	[3]	Brazil
		Best supportive care (BSC)	3 (10)		57	30-71			
1995	Pyrhönen *et al*	5-FU/Epirubicin/Methotrexate (FEMTX)	12.3 (21)	FEMTX	58	42-75	No information	[5]	Finland
		BSC	3.1 (20)		58	42-71			
1997	Glimelius *et al*	5-FU/Leucovorin/Etoposide (FLE)	8 (31)	ELF	64	45-75	No information	[4]	Sweden
		BSC	5 (31)		63	40-74			
1997	Webb *et al*	EPI/CDDP/5-FU (ECF)	8.9 (111)	ECF	59	35-79	No information	[8]	UK
		FAMTX	5.7 (108)		60	29-78			
2000	Vanhoefer *et al*	FAMTX	6.7 (133)	Similar efficacy	58	30-74	No information	[44]	EORTC
		ELF	7.2 (132)		59	25-74			
		5-FU/CDDP (FUP)	7.2 (134)		57	24-74			
2003	JCOG9205	5-FU	7.1 (105)	5-FU	63	27-75	No information	[14]	Japan
		5-FU/CDDP (CF)	7.3 (105)		63	19-75			
		UFT/MMC	6 (70)		60.5	30-75			
2006	V325	Docetaxel/CDDP/5-FU (DCF)	9.2 (227)	DCF	55	26-79	24%-25%[1]	[10]	USA
		CDDP/5-FU (CF)	8.6 (230)		55	25-76			
2007	JCOG9912	S1	11.4 (234)	S1	64	39-75	No information	[17]	Japan
		5-FU	10.8 (234)		63	24-75			
		CPT11/Docetaxel	12.3 (236)		63	32-75			
2008	SPIRITS	S1	11 (150)	S1/CDDP	62	28-74	17%	[18]	Japan
		S1/CDDP (CS)	13 (148)		62	33-74			
2008	REAL-2	EPI/CDDP/capecitabine (ECX)	9.9 (241)	EOX	64	25-82	No information	[9]	UK
		ECF	9.9 (249)		65	22-83			
		EPI/oxaliplatin/capecitabine (EOX)	11.2 (239)		62	25-80			
		EPI/oxaliplatin/5-FU (EOF)	9.3 (235)		61	33-78			
2008	Al-Batran *et al*	5-FU/LV/Oxaliplatin (FLO)	10.7 (112)	FLO	64	33-86	41%-45%[2]	[23]	AIO
		5-FU/LV/CDDP (FLP)	8.8 (108)		64	27-85			

[1]This is proportion of patients with 65 years old or over; [2]This is proportion of patients over 65 years old.

However, most of the cases registered in the INT-116 study were cases that had undergone D0 or D1 surgery, with only 10% of the cases having undergone D2 surgery. The cases registered in the Magic trial, however, also included cases of lower esophagus cancer, cases that could not undergo surgery, cases that ultimately underwent a noncurative resection, and cases of D1 surgery. In addition, the rate of completion of postoperative chemotherapy was low and the efficacy of postoperative adjuvant chemotherapy remained unclear. It was therefore determined that these results could not be applied to Japan, where D2 surgery is a standard procedure, and it was deemed necessary to examine effective adjuvant chemotherapy treatments through comparative studies using a control group of patients who underwent surgery only[33]. In the ACTS-GC, which was started in 2001, the usefulness of postoperative adjuvant S-1 therapy was examined, and interim analysis results indicated that overall survival in the surgery + S1 group was significantly better than in the surgery alone group[34]. Based on these results, postoperative adjuvant chemotherapy with the administration of S-1 alone is considered to be a standard option in Japan. In addition, in the AGTS-GC approximately one-fourth of the registered cases were elderly subjects aged 70 to 80 years old (Table 3). In a subgroup analysis, the administration of S-1 showed significant effects for younger cases aged under 60 years old, but there were no statistically significant better therapeutic effects in cases aged 60 or older. A similar tendency has also been observed in the Magic trial[31]. The proportion of elderly patients in recent milestone phase Ⅲ clinical trials on adjuvant therapy around the world is shown in Table 3. It may be useful to consider this information in making decisions on elderly patients continuing adjuvant chemotherapy if patients have severe side effects.

The INT-116 study found that in cases undergoing radiation chemotherapy, hematotoxicity exceeding grade 3 was observed in 54% of the cases and digestive toxicity was observed in 33%, while in cases undergoing postoperative ECF therapy in the Magic trial, neutropenia exceeding grade 3 was observed in 28% of the cases, leukopenia in 17% of the cases, nausea in 12% of the cases, and emesis in 10% of the cases. On the other hand, in the ACTS-GC, for cases with toxicities exceeding grade 3 due to S-1 therapy, loss of appetite was observed in only 6% of the cases, nausea in 3.7% of the cases, and diarrhea in 3.1% of the cases, and hematotoxicity was mild. In areas where D2 surgery is not a standard procedure, the results of this study are not directly applicable. For example, we have to allow for differences of overall survival in the surgery alone group of the 3 trials; i.e. 3-year survival of ACTS-GC trial (70.1%), 3-year survival of INT 0116 trial (41%), and 5-year survival of MAGIC trial (23%), in

Table 3 Phase Ⅲ milestone trials of adjuvant therapy for gastric cancer after surgical resection and information of elderly patients

Publication	Trials	Patient eligibility	Successful regimens as adjuvant therapy	Adjuvant effect	Median	Range	Proportion of elderly patients (70 years old or over)	Ref.	Country
2001	INT-116	Pathological stage ⅠB/Ⅱ/Ⅲ/ⅣM0	Surgery + Chemo (5-FU/LV)-Radiation (45Gy)	9% at 3 years	60	25-87	No information	[31]	USA
			Surgery alone		59	23-80			
2006	MAGIC	Clinical stage Ⅱ or more (M0)	Surgery + perioperative EPI/CDDP/5-FU (ECF)	13.6% at 5 years	62	29-85	20.4%	[32]	UK
			Surgery alone		62	23-81	21.3%		
2007	ACTS-GC	Pathological stage Ⅱ/ⅢA/ⅢB	Surgery + S1	10% at 3 years	63	27-80	25.9%	[34]	Japan
			Surgery alone		63	33-80	22.6%		

order to interpret the survival data. However, if treatment including locally controlled surgery for gastric cancer is to be performed, postoperative adjuvant chemotherapy with S-1 therapy may be an optimal treatment method that can provide lower toxicity and effects for extending a patient's survival time.

CLINICAL POTENTIAL AND FUTURE PROSPECTIVE FOR ADVANCED GASTRIC CANCER IN ELDERLY PATIENTS

There has been no meta-analysis describing the effect of age on chemotherapy in gastric cancer, however, several subset analyses of the phase Ⅲ milestone trials elucidated the effect of age on chemotherapy, such as both the SPIRITS trial[18] and ACTS-GC[34], and recently emerging AIO study examining both FLO versus FLP[23].

The former two trials conducted in Japan demonstrated that S1 as an adjuvant therapy is more effective in younger patients than the elderly, while CDDP may not add benefits for elderly patients with far advanced gastric cancer. These results suggest that the concomitant use of CDDP is not very effective for elderly patients and that S-1 monotherapy might be the best standard therapy for elderly patients. In completely resected, non-small cell lung cancer (NSCLC), however, the effect of adjuvant CDDP-based chemotherapy showed that it should not be withheld from elderly patients with NSCLC because it was deemed similarly effective in elderly patients as younger patients (hazard ratio, 0.87; 95% CI, 0.68-1.11; test for trend: $P = 0.42$)[35]. These results were different from those of far advanced gastric cancer. Additional CDDP is actually shown to increase hematological and gastrointestinal toxicities in the SPIRITS trials[18], in which grade three-fourths neutropenia (16%), anemia (6%), and thrombocytopenia (0%) in S1 administered patients were increased to 59%, 38%, and 8% in CS therapy, respectively. Furthermore, grade three-fourths anorexia (9%) and nausea (2%) recognized in S1 monotherapy were increased up to 45% and 17% in CS therapy, respectively. Nevertheless, such side effects cannot be a reason for CDDP administration to be withheld in elderly patients if it is effective for far advanced gastric cancer, such as adjuvant administration of CDDP against NSCLC. Further confirmation is needed. On the other hand, newly emerging oxaliplatin as a regimen in the Western world seems to be more effective than CDDP, if limited to elderly patients, suggesting that oxaliplatin might be one of the most recommended regimens for the elderly at present because it also reduced toxicity as compared with CDDP[23].

In analyzing the surgical outcomes of patients with gastric cancer who had undergone a radical operation with D2 dissection, we have shown that the outcome for gastric cancer in elderly patients (aged 60 years old or older) was poorer than younger patients (aged under 60 years old), and the prognostic factor of age was completely independent of cancer progression, even after adjustment for the low degree of lymph node dissection during surgery or differences in the frequency of blood transfusion[36,37]. More intriguingly, diffuse type gastric cancer, which is S-1 sensitive[34], tended to show great differences in prognosis between elderly and young patients, putatively reflecting peritoneal immunity[38]. That is, the poor prognoses for gastric cancer in elderly patients is associated with unspecified factors that cannot be explained with malignancy factors covered by the TNM classification. Candidate factors might include the cancer immunocompetence of the host, since cancer immune system function is well known to deteriorate in the elderly[39], and in the future we can expect the possibility of treatment therapies that take into account the decreased immunocompetence of the host for elderly patients with gastric cancer.

It is believed that the lethal effects of cancer chemotherapy against cancer cells are not simply direct effects but are also affected by the induced immunity derived from dead cancer cells (enhanced cellular immunity due to activated cancer antigens). Considering that adjuvant effects against gastric cancer were stronger in cases of gastric cancer in younger patients in the ACTS-GC[34] and the Magic trial[32], additional treatments that provide improvement of immunity may be important for elderly patients. In a well-controlled, prospective phase Ⅱ trial, it has been shown that Krestin (PSK), which reduces TGF-β in a host and improves cancer immunity[40,41], drastically improves the prognoses of patients with gastric cancer after a radical operation when it is used concomitantly with 5-FU[42]. This suggests that curative effects for cancer may be enhanced by combining S-1 with Krestin, and the efficacy of such combinations may

be expected to be higher in elderly diffuse-type gastric cancer patients. Currently, there is a multi-institutional, prospective, randomized trial being conducted to examine the possibility of using S-1/Krestin and S-1 monotherapy as postoperative adjuvant chemotherapy in gastric cancer (HKIT-GC)[43]. The results of this study, which includes elderly patients, are eagerly anticipated.

CONCLUSION

Regarding chemotherapy treatment for gastric cancer in elderly patients, it is believed that it can be applied as a standard procedure as long as the patient's general health conditions are good and organ functions are sufficiently maintained. However, it is necessary to take into consideration potential deterioration in organ function caused by aging, while sufficient care must be provided in the follow-up stages of treatment. In addition, because elderly patients are nearing the end of their lives, they may have a different sense of priorities compared to younger or middle-aged patients, and it is necessary to fully understand and take into consideration the views of these patients when selecting a treatment method. To improve the treatment results of gastric cancer in elderly patients, we may expect the development of treatment methods that take into consideration modification of unspecified factors, such as cancer immunocompetence, in the future.

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ORIGINAL ARTICLE

Clinical value of ^{18}F-FDG PET/CT in assessing suspicious relapse after rectal cancer resection

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Abstract

AIM: To evaluate the value of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) in the restaging of resected rectal cancer.

METHODS: From January 2007 to Sep 2008, 21 patients who had undergone curative surgery resection for rectal carcinoma with suspicious relapse in conventional imaging or clinical findings were retrospectively enrolled in our study. The patients underwent 28 PET/CT scans (two patients had two scans, one patient had three and one had four scans). Locoregional recurrences and/or distant metastases were confirmed by histological analysis or clinical and imaging follow-up.

RESULTS: Final diagnosis was confirmed by histopathological diagnosis in 12 patients (57.1%) and by clinical and imaging follow-up in nine patients (42.9%). Eight patients had extrapelvic metastases with no evidence of pelvic recurrence. Seven patients had both pelvic recurrence and extrapelvic metastases, and two patients had pelvic recurrence only. ^{18}F-FDG PET/CT was negative in two patients and positive in 19 patients. ^{18}F-FDG PET/CT was true positive in 17 patients and false positive in two. The accuracy of ^{18}F-FDG PET/CT was 90.5%, negative predictive value was 100%, and positive predictive value was 89.5%. Five patients with perirectal recurrence underwent ^{18}F-FDG PET/CT image guided tissue core biopsy. ^{18}F-FDG PET/CT also guided surgical resection of pulmonary metastases in three patients and monitored the response to salvage chemotherapy and/or radiotherapy in four patients.

CONCLUSION: ^{18}F-FDG PET/CT is useful for evaluating suspicious locoregional recurrence and distant metastases in the restaging of rectal cancer after curative resection.

Key words: ^{18}F-fluorodeoxyglucose; Positron emission tomography/computed tomography; Rectal cancer; Follow-up; Restaging; Locoregional recurrence; Distant metastases

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INTRODUCTION

Rectal cancer is one of the most common malignancies in the United States and one of the most frequent causes of cancer-related death[1]. The incidence of rectal cancer is rapidly increasing with changes in life styles and diets and is thus an emerging health issue in the Asian region, including Korea, Japan and China[2,3]. Over the past decade, significant progress has been made in the treatment of localized rectal cancer by advances in surgery, radiotherapy, and chemotherapy. However, distant metastasis and local recurrence continues to be a major problem following treatment of rectal cancer, and carries an extremely poor prognosis[4,5]. More importantly, distant metastasis and locoregional recurrence are seldom curable and produce debilitating symptoms that are difficult to palliate.

Endoscopic examination, conventional computed tomography (CT), and magnetic resonance imaging (MRI) are the main techniques used to follow-up of resected rectal cancer[6,7]. However, these techniques may be not reliable for detecting local recurrence and distant metastasis[8]. The reported increase in sensitivity of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) over CT and MRI has been attributed to the ability of ^{18}F-FDG PET to detect metabolic abnormalities that precede the morphological changes seen by CT[9]. The whole body imaging of the ^{18}F-FDG PET/CT study also contributes to the increased sensitivity through detection of distant metastatic lesions[10]. This study was undertaken to further define the utility of ^{18}F-FDG PET/CT imaging in evaluating suspicious locoregional recurrence and distant metastases in the restaging of resected rectal cancer.

MATERIALS AND METHODS

Patients

In the period from January 2007 to July 2008, 21 patients (14 males and 7 female; age range, 27-78 years; mean age, 58.3 years), who had undergone curative surgery resection for rectal carcinoma and who had suspicious local recurrence and/or distant metastasis in conventional imaging or clinical findings, were retrospectively enrolled in our study. Twenty-one patients underwent 28 PET/CT scans (2 patients had two scans, one patient had three scans and one patient had four scans). The standard of reference for tumor recurrence consisted of histopathological confirmation or clinical and imaging follow-up information for at least 11 mo after PET/CT examinations.

^{18}F-FDG PET/CT technique

The patients were asked to fast for at least four hours before undergoing ^{18}F-FDG PET/CT to ensure that their blood glucose level was within the normal range (70-120 mg/dL) prior to intravenous injection of ^{18}F-FDG. The patients received an intravenous injection of 370-666 MBq (10-18 mCi) of ^{18}F-FDG. Data acquisition by an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA) was performed within 60 min after injection. The procedure of data acquisition was as follows: CT scanning was performed first, from the head to the pelvic floor, with 110 kV, 110 mA, a tube rotation time of 0.5 s, a 3.3-mm section thickness, which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan that covered the identical transverse field of view was obtained. Acquisition time was three minutes per table position. PET image data sets were reconstructed iteratively by applying the CT data for attenuation correction, and coregistered images were displayed on a workstation.

PET/CT image interpretation

Reviewer 1 and reviewer 2, who were aware of other clinical or imaging information, read the ^{18}F-FDG PET/CT images on a high-resolution computer screen. The reviewers reached a consensus in cases of discrepancy. Reviewer 1 had 20 years of experience in both nuclear medicine and radiology, and reviewer 2 had 5 years of experience in both nuclear medicine and radiology. On the basis of knowledge of the normal biodistribution of ^{18}F-FDG, lesions were identified as foci with increased tracer accumulation relative to that in comparable normal contralateral structures and surrounding soft tissues. The lesions were qualitatively graded as definitely or probably abnormal (categorized as representing a tumor) if the accumulation of ^{18}F-FDG was markedly to moderately increased. Diffuse mildly increased activity or no increased activity (in the case of an abnormality identified on CT for which no corresponding abnormality was present on PET) was considered to be normal or benign disease.

RESULTS

Clinical presentation of recurrent disease

Mean time after treatment to PET/CT exam was 17.1 mo (1-51 mo) and mean follow up time after PET/CT exam was 11 mo (1-19 mo). At the time of recurrent rectal cancer being suspected, the mean patient age was 58.3 years with a tendency towards male gender distribution (66.7%). Suspicious locoregional recurrence and distant metastasis for examination with ^{18}F-FDG PET/CT were an unexplained increase in carcinoembryonic antigen values (CEA) (n = 4), unexplained anal pelvic pain (n = 3), suspected pelvic recurrence and distant metastases at CT and MRI (n = 7), suspected pelvic recurrence at colonoscopy (n = 2), or suspected extrapelvic recurrence in follow-up (n = 5).

Locoregional recurrence and distant metastases

Final diagnosis was confirmed by histopathological evidence in 12 patients (57.1%) and by clinical follow-up in nine patients (42.9%). Eight patients (38.1%) had extrapelvic metastases with no evidence of pelvic recurrence. Seven patients (33.3%) had both pelvic recurrence and extrapelvic metastases, and two patients (9.5%) had pelvic recurrence only. ^{18}F-FDG PET/CT was negative in two patients and positive in 19 patients. ^{18}F-FDG PET/CT was true positive in 17 (80.9%)

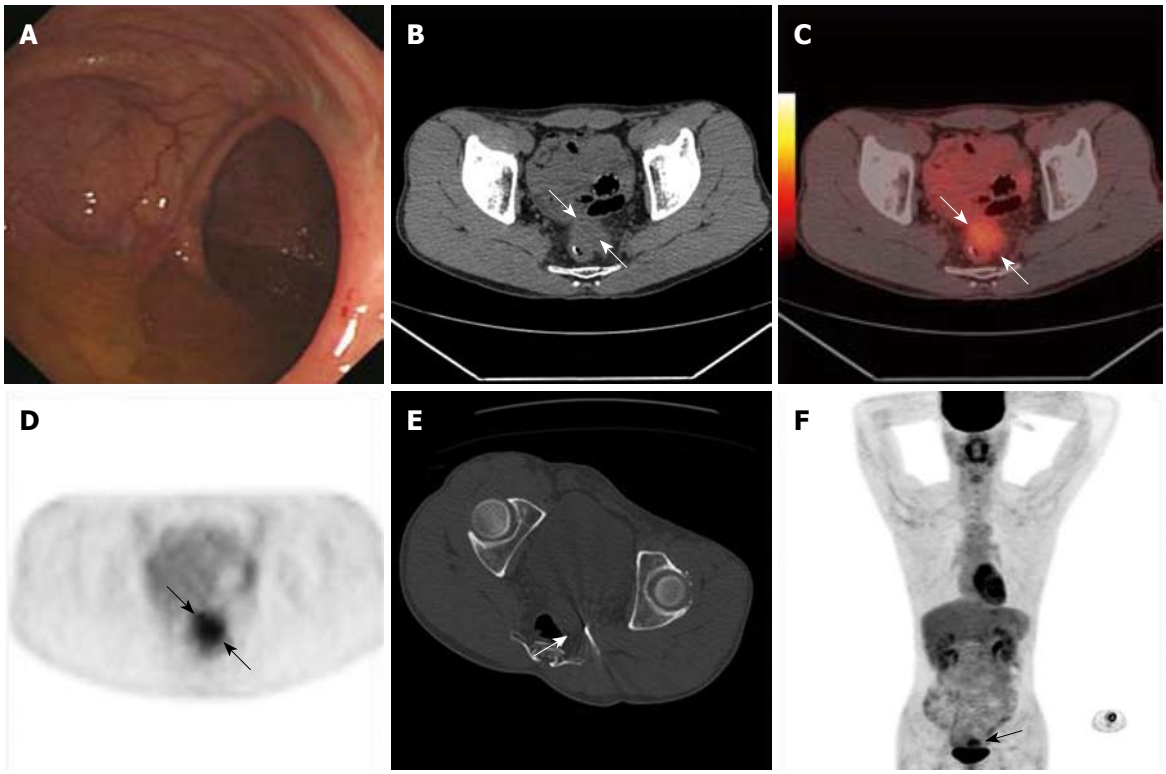

Figure 1 Perirectal recurrence. Endoscopic examination, transverse images and PET/CT images obtained 25 mo after surgery in a 39-year-old man. A: Colonoscopy at the level of tumor resection showed no evidence of recurrence; B: CT demonstrated perirectal soft tissue that might represent a local recurrent tumor or postoperative scar tissue (arrows); C, D: PET/CT revealed a perirectal lesion with high FDG uptake (arrows); E: Local recurrence was confirmed by PET/CT guided tissue core biopsy (arrow); F: Whole body PET/CT confirmed local recurrent tumor (arrow) and no distant metastasis.

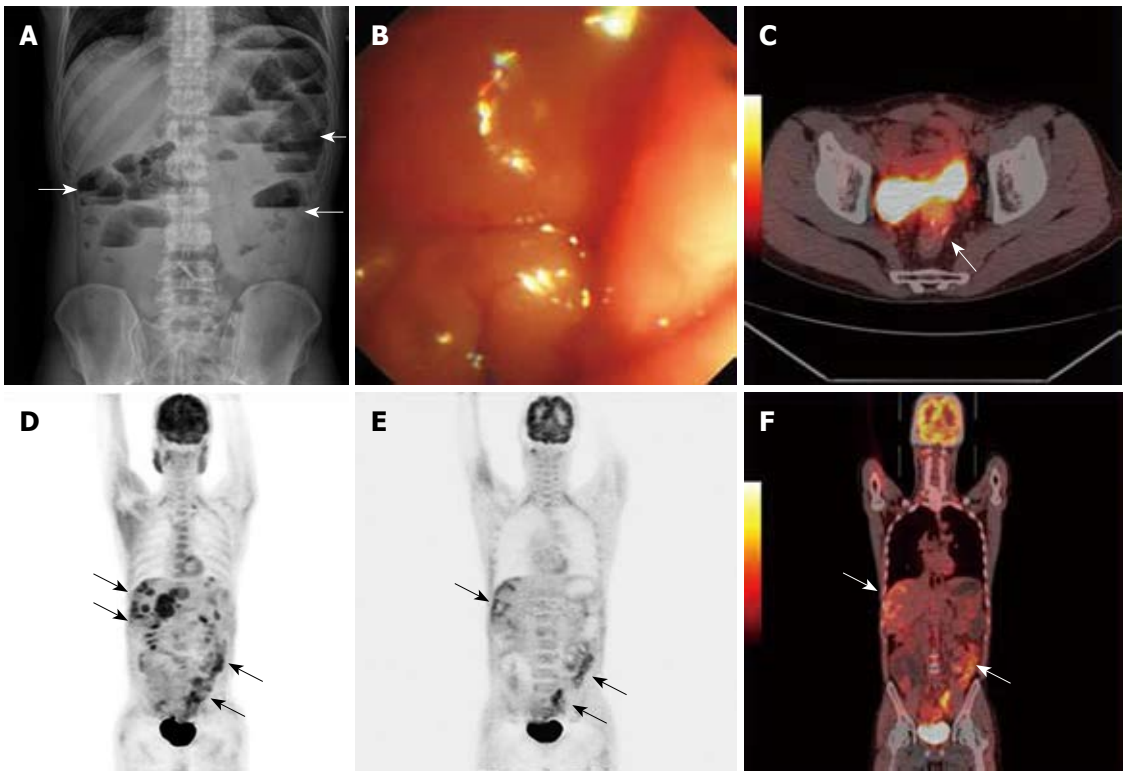

Figure 2 Extrapelvic metastases and perirectal recurrence. Plain abdominal radiograph, ES and PET/CT images obtained 30 mo after rectal cancer resection in a 41-year-old man. A, B: Plain abdominal radiograph and colonoscopy revealed the obstruction at the anastomotic site (arrows); C-F: PET/CT showed perirectal recurrence, peritoneal carcinomatosis and liver metastases (arrows).

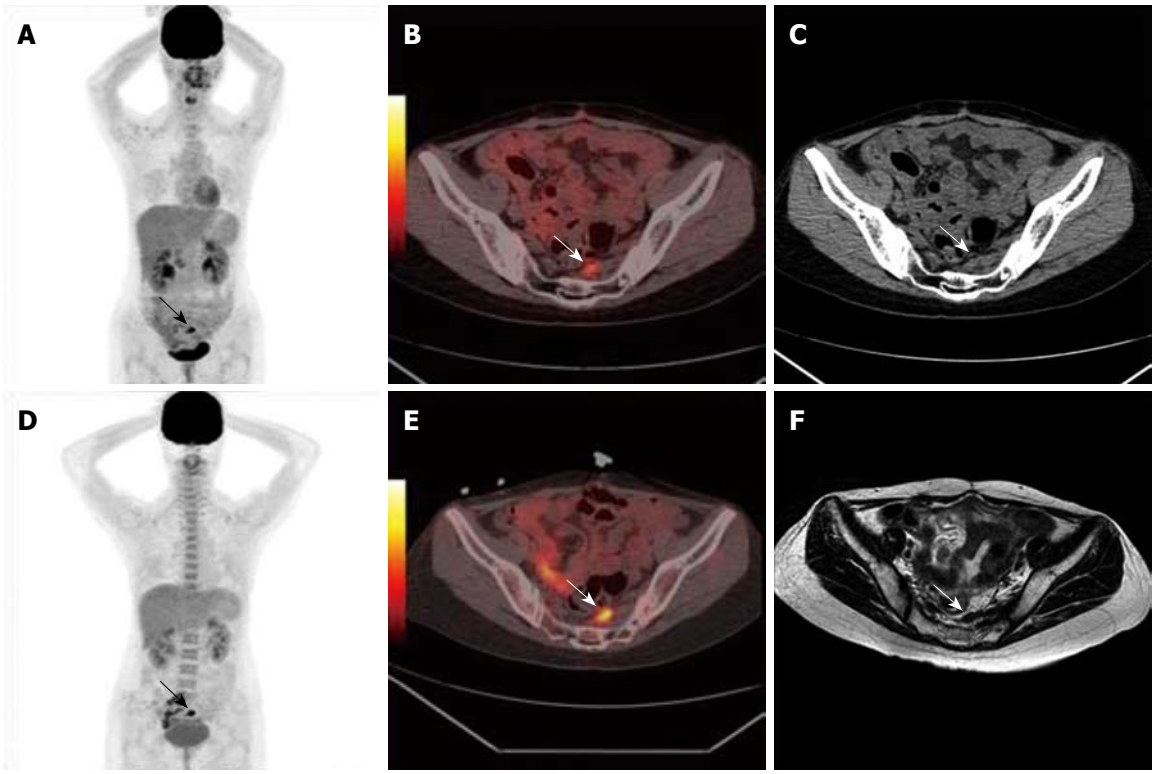

Figure 3 False-positive results. PET/CT obtained in a 34-year-old woman 16 mo after rectal cancer resection. A-C: ^{18}F-FDG uptake in the left side of the pelvis was misinterpreted as tumor recurrence (arrows); D, E: ^{18}F-FDG uptake in the left side of the pelvis was unchanged after 6 mo PET/CT scan (arrows); F: MRI showed no changes of lesion 11 mo follow-up after first PET/CT examination (arrow). The final diagnosis of scar tissue was established by clinical and imaging follow-up.

Table 1 Frequency of locoregional recurrence and distant metastases

Group	Location of recurrences	Frequency *n*
Pelvic recurrence group	Pelvic side walls nodes metastases	1
	Perirectal recurrence	1
Pelvic recurrence and extrapelvic metastases group	Perirectal recurrence	6
	Pelvic side walls nodes metastases	3
	Retroperitoneal lymph nodes metastases	4
	Abdominal wall metastasis	1
	Inguinal lymph nodes metastases	2
	Neck lymph nodes metastases	2
	Liver metastases	2
	Spleen metastases	1
	Lung metastases	4
	Canalis spinalis metastasis	1
	Bone metastases	2
	Peritoneal carcinomatosis	1
Extrapelvic metastases without pelvic recurrence group	Retroperitoneal lymph nodes	2
	Neck lymph nodes metastases	1
	Left axillary nodes	1
	Liver metastases	2
	Lung metastases	4
	Bone metastases	3
	Peritoneal carcinomatosis	1
	Abdominal wall metastasis	1

patients (Figures 1 and 2) and false positive in two (9.5%). Overall, the accuracy of ^{18}F-FDG PET/CT was 90.5%, the negative predictive value was 100%, and the positive predictive value was 89.5%. The two false positive PET/CT findings were inflammation of retroperitoneal abscess and scar tissue in one patient, respectively (Figure 3). 90% of recurrence after curative resection occurs within the first 2 years. Fewer than 9% of recurrences occur after 2 years. Recurrent disease is frequently both pelvic recurrence and extrapelvic metastases (Table 1).

Salvage treatment guided by ^{18}F-FDG PET/CT image

Surgical resection of pulmonary metastases was performed for potentially curative treatment in three patients who had previously undergone resection for rectal carcinoma with a single and peripheral new lung lesion identified by whole body ^{18}F-FDG PET/CT. A one-year follow-up period of the three patients revealed that two of the patients were in good condition and new lung lesion had appeared in one patient five months after the resection of lung metastases. ^{18}F-FDG PET/CT image also was also used to monitor salvage chemotherapy in four patients after two cycles of treatment and revealed the early response to the treatment in these patients (Figure 4).

CT guided tissue core biopsy modulated with ^{18}F-FDG PET/CT image

Five patients who suffered from perirectal recurrence and endoscopic examination of whom failed to get samples for histopathological diagnosis, underwent CT guided tissue core biopsy modulated with ^{18}F-FDG PET/CT imaging (Figure 1). ^{18}F-FDG PET/CT scan and biopsy procedure were completed in each patient with the same PET/CT system on a single day. These patients avoided exploratory laparotomy to obtain samples for histopathological evidence of recurrence.

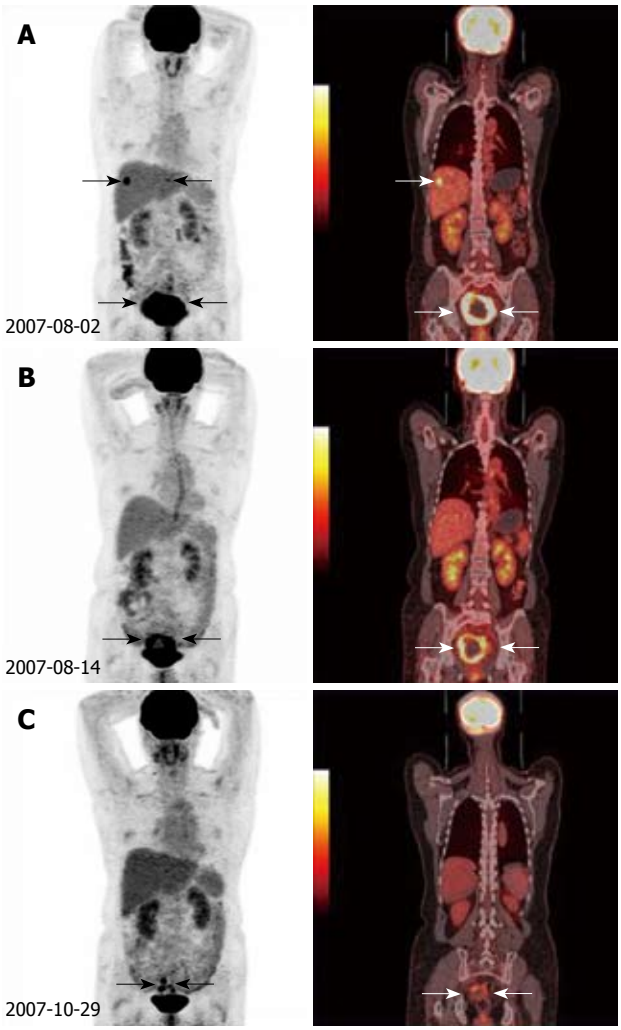

Figure 4 Monitoring responses to pelvic recurrence and liver metastases. Three PET/CT scans were obtained in a 55-year-old woman five months after rectal cancer resection. A: PET/CT demonstrated a locoregional recurrence and liver metastasis foci (arrows) before salvage treatment; B: PET/CT showed obvious decreasing of intense activity after two cycles of chemotherapy (arrows); C: After six cycles of chemotherapy, PETCT imaging follow-up revealed good control of pelvic recurrence and liver metastases (arrows).

DISCUSSION

The frequency of local recurrence after resection of rectal carcinoma is not negligible. Locoregional recurrence after resection of rectal carcinoma is a difficult clinical problem that adversely affects both survival and quality of life[11]. Following potentially curative standard/conventional surgical resection for adenocarcinoma of the rectum, the incidence of locoregional or distant treatment failure is related to the extent of transmural disease and the associated involvement of regional lymph nodes in metastases[12-14]. The incidence of locoregional failure is 8% to 21% in American Joint Committee on Cancer (AJCC) stage I disease, 29% to 44% in AJCC stage II disease, and 50% to 61% in AJCC stage III disease. The incidence of distant failure (as a component of failure) is up to 28% in AJCC stage I disease, 47% in AJCC stage II disease, and up to 74% in AJCC stage III disease[15-17]. However, surgical salvage of locoregional recurrence and distant metastases can be performed safely and can result in substantial long-term survival benefits for selected patients[18,19].

The current treatment strategy for rectal cancer aims detecting locoregional recurrence early by conventional medical modalities. Endoscopic examination is not always useful in such cases, because local recurrence of rectal cancer is often perirectal[20]. In our study, 10 patients had accepted endoscopic examinations one month before ^{18}F-FDG PET/CT scan, which only revealed two suspicious locoregional recurrences. Conventional CT and MRI are also not reliable for detecting local recurrence in some clinical situations, because benign postoperative scar tissue can produce a mass that mimics recurrence[21,22]. Conventional anatomical methods for tumor detection might not accurately distinguish benign and malignant processes based on size criteria alone, and interpretation might be difficult when prior surgery or radiation therapy results in distortion of the normal anatomy[23]. In the current study, 15 patients underwent locoregional CT or MRI scan one month before the ^{18}F-FDG PET/CT scan, which only revealed seven patients with suspicious local recurrences or distant metastases.

The major advantages of ^{18}F-FDG PET/CT are the ability to perform full body examinations, the potential to detect locoregion recurrence and distant metastases in one single examination, and the possibility of distinguishing new active disease from scar or necrotic tissue[24,25]. It has been reported that PET/CT using the radiolabeled glucose analog ^{18}F-FDG was valuable in detecting locoregional recurrence of colorectal cancer, particularly when anatomic imaging modalities have presented equivocal interpretations[26]. According to our results, early detection of extrapelvic metastases is also very important. Our data demonstrated that only 9.5% (2/21) patients had pelvic recurrence, 38% (8/21) patients had extrapelvic metastases with no evidence of pelvic recurrence and 33.3% (7/21) patients had both pelvic recurrence and extrapelvic metastases. In contrast to the results of endoscopic examination and CT/MRI scans, whole body ^{18}F-FDG PET/CT is useful for the early detection of locoregion recurrence and extrapelvic metastases of resected rectal carcinoma, which are the key factors influencing salvage treatment plans for the candidates.

Salvage treatment for the relapse of rectal cancer consisted of radiotherapy alone or combined with chemotherapy and/or surgical resection[27]. Note that ^{18}F-FDG PET/CT could be the best criterion for choosing individual salvage treatment plans[28,29]. Resections are effective for some patients with hepatic or pulmonary metastases of colorectal cancer, but the best selection criteria for resections and the effective treatment for recurrence after the resections have not been determined[30]. However, identifying candidates for this aggressive surgical approach is controversial, because the tumor and host factors that allow systemic disease to be controlled with local therapy are unknown[31]. ^{18}F-FDG PET/CT provides important additional information in patients with presumed resectable colorectal metastases to the liver, leading to a change of therapy in one fifth of patients[32,33].

In our study, surgical resection of pulmonary metastases was used in three patients who had previously undergone resection for rectal carcinoma with a single and peripheral new lung lesion identified by whole body PET/CT. A one-year follow-up period revealed that two of the patients were in a good condition but that a new lung lesion had appeared in the third patient five months after the resection of lung metastases. ^{18}F-FDG PET/CT image was used to guide chemotherapy and radiotherapy in four patients who suffered pelvic recurrence and extrapelvic metastases. Our data, although in a limited series of patients with a relatively short follow-up, showed that the use of whole body PET/CT scan will be helpful for making salvage treatment plans.

Five patients suffered from perirectal recurrence and endoscopic examination failed to get samples for histopathological diagnosis. In such clinical conditions, minimally invasive image-guided biopsy might be the first choice. CT guided biopsy is the most common technique for pathological diagnosis[34]. CT guidance is favorable in small lesions, has reliable three-dimensional control of the biopsy needle path, and, with contrast enhancement, CT can allow discrimination of lesions and vital anatomic vascular and neural structures. However, CT may not accurately distinguish benign postoperative scar tissue and local recurrence based on anatomical characters alone[35]. We performed CT guided tissue core biopsy modulated with ^{18}F-FDG PET/CT imaging in five patients, which might have improved the diagnostic ability of PET/CT, especially when a definite diagnosis was difficult to make. This diagnostic algorithm has the possibility to complete diagnosis and staging of malignant solid tumors in one day.

Our study had several limitations. The first was our small sample size, which might have limited the statistical significance of the results. Due to the retrospective nature of the study, we did not get all of the medical details for some of the patients. There are also some disadvantages associated with PET/CT imaging[36,37]. For example, small tumors might go undetected because partial-volume effects result in a falsely low measurement of true ^{18}F-FDG activity. Another drawback of PET/CT is that ^{18}F-FDG frequently accumulates in areas of inflammation. In our study, the two false positive PET/CT results were inflammation of retroperitoneal abscess and anastomotic granulation tissue, respectively. Physiological variants and benign pathological causes of ^{18}F-FDG uptake can be specifically recognized and properly categorized in other instances.

It is possible that ^{18}F-FDG PET/CT scanning can make a valuable contribution in the detection of locoregional recurrence and distant metastases after rectal cancer resection. The use of ^{18}F-FDG PET/CT might assist the clinician in several ways: such as by guiding surgical resection of hepatic or pulmonary metastases; by improving treatment plans for salvage chemotherapy and radiotherapy; by monitoring the early response to salvage treatment; and by guiding biopsies of suspicious lesions on CT and endoscopic examinations. Our results support the routine use of ^{18}F-FDG PET/CT to evaluate suspicious locoregional recurrence and distant metastases in the restaging of rectal cancer after curative resection.

COMMENTS

Background

The paper tries to evaluate the value of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) in the restaging of resected rectal cancer. They report an accuracy of ^{18}F-FDG PET/CT of 90.5%, a negative predictive value of 100% and a positive predictive value of 89.5%, when used in patients with rectal cancer to evaluate locoregional recurrence. They conclude that ^{18}F-FDG PET/CT is useful as a routine technique to evaluate suspicious locoregional recurrence and distant metastases in the restaging of rectal cancer after curative resection. The study was well designed and performed and appropriate methods were used, however, some changes would help to improve the manuscript

Innovations and breakthroughs

The authors use ^{18}F-FDG PET/CT in 21 patients. They carried out one scan for all patients and up to four scans for one patient who had undergone curative surgical resection for rectal carcinoma to determine suspicious relapse. They demonstrated the usefulness of this technique to evaluate recurrences and/or distant metastases. Also, they confirmed the results with histological analysis or clinical and imaging follow-up.

Applications

This is a powerful technology for tumor visualization, staging and recurrence diagnosis.

Peer review

This is an interesting article on the application of PET-CT to the study of the relapse after rectal cancer resection. The conclusions are sound and might be of interest for the clinical community.

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BRIEF ARTICLE

Vascular endothelial growth factor, *p53*, and the *H-ras* oncogene in Egyptian patients with bladder cancer

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Abstract

AIM: To evaluate the relationship between vascular endothelial growth factor (VEGF), *p53*, and the *H-ras* oncogene and different clinicopathological parameters in Egyptian patients with Schistosoma-associated transitional cell carcinoma of the bladder.

METHODS: The study included 50 patients with transitional cell carcinoma for whom radical cystectomy and urinary diversions were carried out. VEGF and p53 protein expressions were evaluated with an immunohistochemical staining method, and *H-ras* oncogene mutations were analyzed with a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

RESULTS: High grade tumors revealed higher p53 immunostaining than low grade tumors (P = 0.016). p53 and VEGF protein expressions, as well as *H-ras* oncogene mutations, had an insignificant impact on patient outcomes (P = 0.962, P = 0.791, and P = 967, respectively). Cancer extension to regional lymph nodes was associated with poor outcomes (P = 0.008).

CONCLUSION: VEGF, *p53* and the *H-ras* oncogene have no relation to patient survival and outcome in Schistosoma-associated transitional cell carcinoma.

Key words: Bladder cancer; Transitional cell carcinoma; Vascular endothelial growth factor; *p53*; *H-ras* oncogene

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INTRODUCTION

Carcinoma of the bladder is the most common cancer of the urinary tract. It is the most common solid tumor in men in Egypt. Squamous cell carcinoma accounted for 59.3% of cases, transitional cell carcinoma for 22.2% of cases and adenocarcinoma for 11.4% of cases in radi-

cal cystectomy specimens studied at the Mansoura Urology and Nephrology Center[1]. Squamous cell carcinomas are common due to associated schistosomiasis[2].

To date, there is no reliable method to identify those patients at risk for recurrence and those who will develop more aggressive disease[3]. The histopathological parameters, such as tumor grade, stage, and lymph node involvement, are the most important parameters in evaluation of the biological behavior of bladder cancer[1,4].

However, there are multiple studies that have shown that clinical, as compared to pathological staging, is inaccurate[5]. The overall error in clinical staging was 32.9%, and, in the majority of cases, the error was due to clinical underestimation of the extent of the disease[1,5]. Therefore, there is continued evaluation of staging systems for bladder cancer due to variable responses of histologically similar tumors to similar treatments[6]. Furthermore, inter- and intra-individual inconsistency seems to be unavoidable in grading bladder tumors[7].

A significant relationship was found between tumor angiogenesis and the presence of lymph node metastasis[8] and prognosis[9,10] in patients with superficial and invasive bladder carcinoma. In contrast, Stavropoulos *et al*[11] found no correlation between angiogenesis and survival of patients with negative lymph node metastasis treated by radical cystectomy. Also, there was no significant correlation between angiogenesis and clinical outcome in patients with stage pT1 disease[12].

The aim of this retrospective study was to clarify the relation between vascular endothelial growth factor (VEGF), *p53*, and the *H-ras* oncogene and different clinical and pathological parameters such as age, sex, histologic cell type, tumor grade, stage, and lymph node status. Also, the goal was to define the relationship between VEGF and p53 in bladder carcinoma that was treated by radical cystectomy and the disease-free survival rate, aiming to detect the significant prognostic factor(s) that can affect patient survival.

MATERIALS AND METHODS

This study was conducted on 50 patients with transitional cell carcinoma for whom radical cystectomy and urinary diversions were performed between years 2002 and 2003. The patients of this study were selected on the basis of the availability of complete archival material. Indications for surgery were failure of endoscopic control of superficial bladder tumors despite adjuvant intravesical chemotherapy and/or immunotherapy, and invasive tumors without evidence of distant metastasis. At the time of cystectomy, all specimens were examined according to the same pathological protocol. Tissue sections were obtained from the tumor, bladder wall, and regional lymph nodes. Tissues were studied from the seminal vesicle and prostate in men and from the ovaries and uterus in women. Tumors were graded using the World Health Organization (WHO) classification[13], and the TNM classification of the International Union against Cancer (UICC) was used for pathological staging of the tumors[14].

VEGF and p53 with an immuno-histopathologic staining technique

Four-micron thick sections from retrieved tumor blocks were placed at 60℃ for 15 min, incubated in xylene at RT for 15 min, and then transferred sequentially into graded alcohols (100%, 95%, 70%, and 50%) for 4 min at RT. Sections were rinsed in deionized water and stored in PBS. Antigen sites were unmasked using a microwave antigen retrieval technique in Coplin jars containing 0.1 mol/L Tris-HCl buffer containing 5% urea, pH 9.0 and heated in a microwave oven for 10 min (two times)[15,16]. After cooling, the slides were rinsed with phosphate buffered saline (PBS). Endogenous peroxidase activity was removed by submersing the slides in blocking solution that was prepared by adding one part of 30% H_2O_2 to nine parts of methanol. Non-specific binding was blocked with blocking serum (reagent A), which was left for 10 min in contact with the slides. After removing the excess blocking serum, the primary monoclonal antibody (Zymed, USA) was applied at a dilution of 1:50 in PBS and incubated for 60 min. The slides were rinsed well with PBS for 3 min. Biotinylated secondary antibody solution (reagent B) was incubated with the slides for 20 min. This was followed by a further wash in PBS. Enzyme conjugate solution (ready to-use streptavidin-peroxidase HRP (Reagent C) (reagent A, B, and C from Histostain®-plus bulk kit, Zymed, USA) was applied to the slides for 10 min. The slides were washed with buffer solution followed by the final steps of this procedure; i.e. addition of chromogen substrate di-amino-benzidine (DAB) (Sigma chemical company, UK) and light counter-stain with hematoxylin.

VEGF and p53 protein expressions were assessed in areas with solid tumor morphology away from any artifact or necrosis, and without prior knowledge of the patient outcome. Assessment of protein expression was performed in three areas where the highest density of expression was found (hot spots). Low power light microscopy at × 40 magnification was used to scan the heterogenous tumor sections for identification of these areas. At × 250 magnification, examination was made in all distinct brown-staining endothelial cells. Protein expression found in unaffected areas, adjacent to tumor infiltrated tissues, was not evaluated, but used as an internal control in assessing quality.

Detection of point mutations at codon 12 of the H-ras oncogene by PCR-RFLP

DNA extraction was done using Clontech kits (UK). The mutant specific PCR-RFLP method is as follows. Amplifications with Taq polymerase were done in 25 μL reaction mixtures containing 21 μL of Taq PCR Master Mix (Qiagen, USA), 2 μL of extracted DNA and 1 μL of each primer (Outer primers A and B). The reaction mixtures were subjected to amplification cycles of 95℃ for 1 min, 57℃ for 1.5 min and 72℃ for 2 min for a total

of 14 cycles. Then, a restriction enzyme was used to cleave the amplified DNA sequence, namely Nae I (its cleavage site is GCCGGC). This restriction enzyme can cleave wild type but not mutant *H-ras* genes. The reaction mixture for the digestion with this restriction enzyme contained 8 μL amplified DNA, 1 μL of restriction enzyme and 2 μL of 10 X buffer and 9 μL of distilled water in a total volume of 20 μL. This reaction mixture was incubated at 37℃ for 2 h. Then, a second PCR of 16 cycles was performed and 2 μL of the first digest was used as a template for the second PCR in which other 2 inner primers, C and D, were used to amplify the non-cleaved mutant sequence. The products of the second PCR were incubated with the restriction enzyme as previously mentioned for 2 h. Finally, the PCR products were resolved by electrophoresis in 2% agarose gels with 120 V for 45 min and were visualized by staining with ethidium bromide. The restriction enzyme-resistant DNA fragment is diagnostic of a mutant sequence at codon 12 of the *H-ras* gene. The primer sequence was list in Table 1.

Follow-up

Patients were kept under regular clinical review. Follow-up data, including mortality data, were obtained for all patients. Patients were followed regularly and examined for treatment failure depending on clinical and radiological findings or histopathological evidence. The mean follow-up was 20.4 ± 8.3 mo for transitional cell carcinoma.

Statistical analysis

The data were initially analyzed by taking one factor at a time and testing for any relationship with survival. Kaplan-Meier survival curves were plotted and log-rank tests were used to determine statistical differences between life table curves[17]. The period of disease-free survival was defined as the time between the date of surgery and death (from cancer) or the development of local recurrence or distant metastasis. Death from unknown cause was considered death from cancer. Censored survival values represent patients who were alive without clinical evidence of disease at the time of last follow-up. To simplify the statistical analysis, the patients were divided into two groups according to their age (≤ 50 years and > 50 years groups) and also, pathologic tumor stage was further subdivided into organ confined tumors (stages pT1 and pT2) and non-organ confined tumors (stages pt3 and pT4). χ^2 tests were performed to evaluate the relationship between p53 as well as VEGF immunostaining, ras gene mutations and different clinicopathological parameters. Statistical analyses were performed using SPSS statistical software packages (SPSS inc., Chicago, IL, USA). $P < 0.05$ was consider significant.

RESULTS

An insignificant relationship was observed between p53 immunostaining and age, sex, stage, and lymph node metastasis. A statistical significance was noticed between p53 immunostaining and tumor grade ($P = 0.016$). Higher grade tumors showed higher expression of p53 protein than low grade tumors (Table 2).

Table 1 Sequence of oligonucleotide primers

Primer	Sequence (5'→3')
Outer primers	
Sense (A)	AGGAGCGATGACGGAATATAAGC
Antisense (B)	GGCTCACCTCTATAGTGGGGTCGTATT
Inner primers	
Sense (C)	AATATAAGCTGGTGGTGGTGGGCGC
Antisense (D)	GGGGTCGTATTCGTCCACAAAATG

Table 2 Clinicopathological characteristics of the transitional cell carcinoma group and their relation to p53 immunostaining *n* (%)

Variable	*n*	P53		*P* value
		Positive	Negative	
Age (yr)				
≤ 50	11 (22)	7 (14)	4 (8)	0.851
> 50	39 (78)	26 (52)	13 (26)	
Sex				
Male	42 (84)	27 (54)	15 (30)	0.558
Female	8 (16)	6 (12)	2 (4)	
Grade				
G2	18 (36)	8 (16)	10 (20)	0.016
G3	32 (64)	25 (50)	7 (14)	
Stage				
pT1	8 (16)	3 (6)	5 (10)	
pT2	28 (56)	21 (42)	7 (14)	0.079
pT3	7 (14)	6 (12)	1 (2)	
pT4	7 (14)	3 (6)	4 (8)	
Node status				
Positive	6 (12)	2 (4)	4 (8)	0.072
Negative	44 (88)	31 (62)	13 (26)	

No correlation was found between VEGF immunostaining, *H-ras* oncogene mutation and age, sex, tumor grade, stage, and lymph node status (Tables 3 and 4).

The 3-year survival rate of the transitional cell carcinoma group was 68%. Most treatment failures occurred during the first two years of follow-up after cystectomy. Node negative patients had better survival compared to those with lymph node metastasis. The 3-year survival rate was 75% for node negative cases, *vs* 16.7% for those with lymph node metastasis. Extension of cancer to the regional lymph nodes was associated with poor outcome ($P = 0.008$).

No impact was noticed for age and sex on patient outcome ($P = 0.487$), however, females had better survival rates than males but without a statistically significant difference ($P = 0.126$). There was no relationship between p53 positivity, VEGF protein expression or the presence of *H-ras* oncogene mutations and patient survival ($P = 0.962$, $P = 0.791$, and $P = 0.967$, respectively; Table 5).

There was a statistically insignificant difference between grades 2 and 3 regarding survival ($P = 0.128$). There was no statistical difference between pT2, pT3, and pT4 tumors regarding prognosis ($P = 0.601$). Further division of transitional cell carcinoma groups into organ-confined (pT1 and pT2; 36 cases) and non-organ confined tumors (pT3 and pT4; 14 cases) was carried out.

Table 3 Clinicopathological characteristics of the transitional cell carcinoma group and their relation to VEGF immunostaining *n* (%)

Variable	*n*	VEGF		*P* value
		Positive	Negative	
Age (yr)				
≤ 50	11 (22)	5 (10)	6 (12)	0.364
> 50	39 (78)	12 (24)	27 (54)	
Sex				
Male	42 (84)	16 (32)	26 (52)	0.161
Female	8 (16)	1 (2)	7 (14)	
Grade				
G2	18 (36)	6 (12)	12 (24)	0.941
G3	32 (64)	11 (22)	21 (42)	
Stage				
pT1	8 (16)	3 (6)	5 (10)	0.935
pT2	28 (56)	9 (18)	19 (38)	
pT3	7 (14)	3 (6)	4 (8)	
pT4	7 (14)	2 (4)	5 (10)	
Node status				
Positive	6 (12)	1 (2)	5 (10)	0.339
Negative	44 (88)	16 (32)	28 (56)	

VEGF: Vascular endothelial growth factor.

Table 4 Clinicopathological characteristics of the transitional cell carcinoma group and their relation to *H-ras* mutations *n* (%)

Variable	*n*	*H-ras* mutation		*P* value
		Positive	Negative	
Age (yr)				
≤ 50	11 (22)	1 (2)	10 (20)	0.479
> 50	39 (78)	7 (14)	32 (64)	
Sex				
Male	42 (84)	7 (14)	35 (70)	0.768
Female	8 (16)	1 (2)	7 (14)	
Grade				
G2	18 (36)	4 (8)	14 (28)	0.368
G3	32 (64)	4 (8)	28 (56)	
Stage				
pT1	8 (16)	1 (2)	7 (14)	0.156
pT2	28 (56)	4 (8)	24 (48)	
pT3	7 (14)	3 (6)	4 (8)	
pT4	7 (14)	0 (0)	7 (14)	
Node status				
Positive	6 (12)	0 (0)	6 (12)	0.254
Negative	44 (88)	8 (16)	36 (72)	

The organ-confined group showed better outcomes than the non-organ confined group, however, this difference did not reach a statistically significant level ($P = 0.3$). The 3-year survival rate for the organ-confined group was 85.7% *vs* 42.8% for the non-organ confined group.

DISCUSSION

Carcinoma of the urinary bladder is the most common solid tumor in men in Egypt. It represents 30.3% of all cancer cases treated at the National Cancer Institute in Cairo[18]. Recently, Gouda *et al*[19] reported a significant decline of the relative frequency of bladder cancer to 11.7%, Bilharzia association from 82.4% to 55.3%, and squamous cell carcinomas from 75.9% to 28.4%. At the same time, this decline was associated with a significant rise in transitional cell carcinomas from 16.0% to 65.8%, an increase in the median age of patients from 47.4 years to 60.5 years, and a decrease of the male: female (M/F) ratio from 5.4 to 3.3.

Table 5 Kaplan-Meier estimates of 3-year disease-free survival in relation to patient and tumour characteristics in the transitional cell carcinoma group

Characteristic	*n*	3-year survival rate (%)	*P* value
Total No.	50	68.0	
Age (yr)			
≤ 50	11	71.8	0.487
> 50	39	54.5	
Sex			
Male	42	64.3	0.126
Female	8	87.5	
Grade			
G2	18	72.2	0.128
G3	32	65.6	
Stage			
pT1	8	100	
pT2	28	71.4	0.601
pT3	7	57.1	
pT4	7	28.6	
Node status			
Positive	6	16.7	0.008
Negative	44	75.0	
p53			
Positive	33	63.6	0.962
Negative	17	76.5	
VEGF			
Positive	17	64.7	0.791
Negative	33	69.7	
H-ras			
Positive	8	66.7	0.967
Negative	42	75.0	

The age range of this study group was 41-87 years (59.0 ± 9.9). In western countries, the median age is 65 years, while in Egyptian series, it was 46 years[19]. However, Koraitim *et al*[20] noticed a marked shift in the age-related incidence curve for Schistosoma-associated bladder carcinoma towards an older age group approximating that in non-Schistosomal cases.

Despite the use of advanced imaging techniques, regional or distant metastases may go unnoticed until surgery or at follow-up. There is ample documentation that clinical compared to pathological staging is inaccurate in one third of cases, with a tendency for underestimation of the extent of the disease. Therefore, there is a need for additional objective information on the aggressiveness of bladder tumors[21].

Several investigators have shown that tumor angiogenesis is important for continued tumor growth and progression. It has been suggested that angiogenic capacity is an early marker of preneoplastic and neoplastic lesions of the human bladder, and the development of a vascular network is integral to tumor progression[10]. The formation of a vascular network is not only essential for tumor growth but also provides a route by which tumor emboli may disseminate, resulting in the development of metastatic disease. There appears to

be a quantitative relationship between angiogenesis and prognosis in several human cancers. Moreover, angiogenic activity was correlated with a higher incidence of lymph node metastasis and poor prognosis for patients with transitional cell carcinoma of the bladder[22,23].

Mutational studies of the *H-ras* gene family have demonstrated that an alteration in codons 12 and 61 of the *H-ras* gene occurs in about 20% of bladder cancers[24].

In Schistosoma-associated bladder carcinoma, the incidence of transitional cell carcinoma ranged from 16% to 43.8%[20]. In western countries, transitional cell carcinoma represents 90% of bladder cancers[25]. In the current study, the overall 3-year survival rate of patients with transitional cell carcinoma was 68%. There was no significant impact of patient age or sex on the patient outcome. Similar results have been previously reported[1,26].

In this study, transitional cell carcinomas were of grade 2 (36%) and grade 3 (64%) because most of the patients had invasive transitional cell carcinoma (84%). This study showed that tumor grade did not significantly affect patient outcomes in transitional cell carcinoma according to univariate analysis. The 3-year survival rate of patients with grade 2 tumors was 72.2% *vs* 65.6% for those with grade 3 ($P = 0.128$). Similar results have been previously reported[26,27]. However, other reports demonstrated a significant relationship between tumor grade and patient survival[1].

These contradictory results might be due to the presence of inter- and intra-individual variations in evaluation of tumor grades in patients with bladder tumors[7,28]. On the other hand, Ghoneim *et al*[1] studied a large cohort of patients (1026 patients; 764 men and 262 women) with different types of bladder cancer (Squamous, transitional carcinoma, and adenocarcinoma), with very long follow up times (up to 24 years) and relative higher incidences of regional lymph nodes metastasis (18.3%).

The critical importance of tumor stage had been recognized in several studies[1,29]. In the present study, there was a tendency of survival advantage in low stage tumors compared to high stage tumors. The 3-year survival rates were 100%, 71.4%, 57.1%, and 28.6% for stage pT1, pT2, pT3, and pT4, respectively. However, these differences in survival were not significant ($P = 0.601$). This insignificant relationship between tumor stage and survival might be attributed to the small number of patients in this group (50 patients) and that most of patients in our study had stage pT2 (56%). The small number of patients in other stages rendered statistical analysis of tumor stage in relation to patient survival insignificant. Also, there was no significant difference between the organ-confined group (pT1 and pT2) and the non-organ confined bladder group (pT3 and pT4) in the 3-year survival group ($P = 0.3$).

The incidence of lymph node metastasis for patients with transitional cell carcinoma was 12%. Lymph node metastasis was associated with significantly poor prognosis. Node negative patients had a 3-year survival rate of 75% in comparison to 16.7% for patients with lymph node metastasis. Similar results were reported in previous studies[29-31].

In the present study, tumor cell surface expression of p53 and VEGF proteins as well as the presence of *H-ras* gene mutations had no prognostic value in Schistosoma-associated transitional cell carcinoma of the bladder. Similar results were previously reported either with invasive bladder tumors[32], or with superficial bladder tumors[12,33,34]. Our results were not in agreement with other reports that found a significant relationship between VEGF and p53 markers and prognosis[35], and lymph node metastasis[8] in bladder cancer.

These contradictory results might be attributed to different study population criteria and different methodology. For example, the Lorenzo-Romero *et al*[35] study was done on 115 samples (21 cases without and 94 cases with bladder tumor). In this study, 63.8% of patients had superficial and 37.2% had infiltrative (20% recurrent) transitional cell carcinoma (16% and 84% in the present work, respectively), p53 detection was done at the level of DNA (not tissue expression) by polymerase chain reaction-single strand conformational polymorphism analysis, and lastly, all the cases of our work were Schistosoma-associated bladder carcinoma.

The difference in results between the present work and that of Suzuki *et al*[8] can be explained by differences in patient numbers (87 *vs* 50), longer follow up times (42 mo *vs* 20 mo), higher percentages of nodal involvement (25% *vs* 12%), and immunohistochemistry detection was done for VEGF-C, which is one of the isoforms of VEGF.

There was no relationship between tumor expression of both p53 and VEGF proteins, as well as the presence of *H-ras* gene mutations, and the different clinicopathologic factors, except for the presence of a significant relation between the expression of p53 and tumor grade ($P = 0.01$). High grade tumors had increased incidence of cell surface expression of p53 compared to low grade tumors. Also, invasive bladder tumors (30 out of 42 cases) demonstrated higher expression of p53 protein than superficial bladder tumors (3 out of 8 cases). The observed difference in surface expression of p53 between superficial low grade and invasive high grade bladder tumors in our study may support the idea that there may be two different angiogenic pathways in bladder cancer that are associated with different tumor morphology and behavior[36]. One pathway is related to the superficial low grade bladder tumors with organized branching and the other to the muscle invasive and solid tumors with disorganized vasculature[12].

With Kaplan-Meier analysis, lymph node status was the only significant prognostic factor in transitional cell carcinoma. Patients with lymph node metastasis had an increased risk to die much higher than those with negative lymph nodes.

In conclusion, our results suggest that the degree of VEGF, p53, and *H-ras* oncogene expression has no relation to patient survival and outcome in Schistosoma-associated transitional cell carcinoma. Invasive high grade bladder tumors had more cell surface expression of p53

protein than superficial low grade bladder tumors. Hence, lymph node metastasis could provide more objective tools for better judgment on the patient survival and help in choosing the most convenient therapy for individual patients. Additional studies, with wider prospective series and longer follow up, should address whether angiogenesis predicts prognosis and recurrence in separate and homogenous samples of patients with bladder tumors that equally reflect all categories.

COMMENTS

Background

Carcinoma of the bladder is the most common cancer of the urinary tract. It is the most common solid tumor in men in Egypt. To date, there is no reliable method to identify those patients at risk of recurrence and those who will develop more aggressive disease. The histo-pathological parameters such as tumor grade, stage, lymph node involvement are the most important parameters in evaluation of the biological behavior of bladder cancer. However, there are multiple documents that clinical as compared to pathological staging is inaccurate.

Research frontiers

In bladder tumor, there was a significant relationship between tumor angiogenesis and the presence of lymph node metastasis, and prognosis in patients with superficial and invasive bladder carcinoma. The research hotspot is how to clarify the relation between certain angiogenic and oncogenic markers [vascular endothelial growth factor (VEGF), *p53*, and *H-ras* oncogene] and risk of recurrence and their significance as prognostic markers.

Innovations and breakthroughs

VEGF and p53 protein expression were evaluated by using immunohistochemical staining method, and H-Ras oncogene mutation by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The study showed that high grade tumors revealed higher p53 immunostaining than low grade tumors. p53, VEGF protein expression as well as H-ras oncogene mutation had insignificant impact on patient outcome and cancer extension to regional lymph nodes was associated with poor outcome.

Applications

Lymph node metastasis could provide more objective tools for better judgment on the patient survival and help in choosing the most convenient therapy for individual patient.

Peer review

In this manuscript, the authors examined vascular endothelial cell growth factor, p53, and H-Ras oncogene as prognostic markers in Egyptian patients with transitional cell bladder carcinoma. They concluded that p53, VEGF, and H-ras mutations have no prognostic value in Schistosoma-associated transitional cell carcinoma of the bladder. Only lymph node status provides prognostic information. In general, the manuscript is reasonably well written and addresses a critical issue, the molecular pathogenesis of Schistosoma-associated bladder cancer.

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BRIEF ARTICLE

Comparison between colorectal low- and high-grade mucinous adenocarcinoma with MUC1 and MUC5AC

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Abstract

AIM: To explore useful prognostic factors for mucinous adenocarcinoma (MAC) in the colon and rectum.

METHODS: MAC was divided into low- and high-grade types based on the degree of structural differentiation; low-grade MAC arisen from well to moderately differentiated adenocarcinoma and papillary carcinoma, and high-grade MAC from poorly differentiated adenocarcinoma and signet ring cell carcinoma. Immunohistochemically, the expression of 2 types of MUC1 (MUC1/DF and MUC1/CORE), MUC2, 2 types of MUC5AC (MUC5AC/CHL2 and HGM), MUC6, CDX2, and CD10 was examined in 16 cases of MAC consisting of 6 low- and 10 high-grade types.

RESULTS: MUC1/DF3 was expressed in 3 of 6 low-grade MAC (50%) and 10 of 10 high-grade MAC (100%). MUC1/CORE was expressed in 1 of 6 low-grade MAC (16.7%) and 7 of 10 high-grade MAC (70%). MUC2 was expressed in all MAC regardless of the grade. MUC5AC was expressed in 6 of 6 low-grade MAC (100%) and 4 of 10 high-grade MAC (40%). HGM was expressed in 5 of 6 low-grade MAC (83.3%) and 6 of 10 high-grade MAC (60%). Expression of MUC6 and CD10 was undetected in all MAC regardless of the grade. CDX2 was expressed in 5 of 6 low-grade MAC (83.3%) and 7 of 10 high-grade MAC (70%). Taken together, MUC1/DF3 was expressed significantly more frequently in high-grade MAC than in low-grade, and MUC5AC/CHL2 was expressed significantly more frequently in low-grade MAC than in high-grade.

CONCLUSION: It is proposed that MUC1/DF3 and MUC5AC/CHL2 immunostaining is useful to discriminate high-grade MAC from low-grade MAC.

Key words: Mucinous adenocarcinoma; Colon; Rectum; MUC1; MUC5AC

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INTRODUCTION

Mucinous adenocarcinoma (MAC) is defined as a carcinoma with mucin composing more than 50% of the

lesion and characterized by pools of extracellular mucin that contain malignant epithelium as acinar structures, strips of cells or single cells. MAC is not classified into subtypes in the World Health Organization Classification of Tumors of the Digestive System[1]. However, it has been reported that MAC can be divided into two types based on the degree of structural differentiation. One type of MAC is low-grade MAC arisen from well to moderately differentiated adenocarcinoma and papillary carcinoma, and the other high-grade MAC arisen from poorly differentiated adenocarcinoma and signet ring cell carcinoma[2].

Mucins are the major component in the mucus gel on epithelial surfaces with a characteristic organ- and cell type-specific distribution. MUC1 is a large cell surface mucin glycoprotein expressed by most glandular and ductal epithelial cells[3]. Normal stomach mucosa is characterized by the production of MUC5AC by the surface epithelial mucus cells and MUC6 by the gastric glands[4]. MUC2 is the secreted mucin present predominantly in small and large intestine and confined to goblet cells[4]. It has also been reported that altered mucin expression is a feature of precancerous and cancer cells. For example, the expression of MUC1 is up-regulated in a variety of carcinomas including colorectal carcinoma[5-8]. A decrease of MUC2 expression has been reported in poorly differentiated colorectal adenocarcinoma[8]. The gastric mucin MUC5AC has been reported to be expressed in colorectal adenocarcinoma[8]. In addition, CD10, a membrane-bound zinc metallopeptidase, is a small intestinal type-brush border marker and is expressed in the intestinal phenotype of gastric carcinoma[9]. The expression of CD10 has not been examined in colorectal MAC. CDX2, an intestine-specific transcription factor, is expressed in the nuclei of normal colorectal tissue, colorectal adenocarcinoma, and mucinous types of adenocarcinoma of ovarian and lung origin[10,11]. However, expression of these molecules has not been investigated to compare low-grade MAC with high-grade MAC. To find useful prognostic factors of colorectal MAC, we here immunohistochemically examine the expression of MUC1, MUC2, MUC5AC, CD10 and CDX2 in relation to the grade of colorectal MAC.

MATERALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All patients provided written informed consent.

Cases

Sixteen patients with colorectal MAC underwent surgical resection at the Hospital of Hyogo College of Medicine between 2001 and 2005. These 16 MAC were divided into 6 low- and 10 high-grade tumors based on the degree of structural differentiation. Each specimen was examined by 2 authors (T.N. and T.T.) with a multiheaded microscope to reach a consensus on pathological diagnosis. Six patients were male and 10 female with a mean age of 62.1 ± 14.5 years (44-90 years) (Table 1).

Immunohistochemistry

The immunohistochemical analysis was carried out according to the method described previously[12]. Briefly, tissues embedded in paraffin were cut into 5-μm-thick sections. The sections were heated in 10 mmol/L sodium citrate buffer (pH 6.0) at 95℃ for 40 min to facilitate antigen retrieval. For immunohistochemical analysis of MUC1, 2 monoclonal antibodies, MUC1/DF3 (15-3, Toray-fuji Bionics, Tokyo, Japan) that identified the core peptide of MUC1 with sialyl oligosaccharides and MUC1/CORE (Ma695, Novocastra Lab, Newcastle Upon Tyne, UK) that recognized the core peptide of MUC1, were used. For immunohistochemical analysis of MUC5AC, MUC5AC/CLH2 (CLH2, Novocastra Lab) that identified only MUC5AC without glycosidic modification and HGM (45M1, Novocastra Lab) that recognized MUC5AC regardless of glycosidic modification were used. The sections were incubated with the primary antibodies of MUC1/DF3, MUC1/CORE, MUC2 (Ccp58, Novocastra Lab), MUC5AC/CHL2, HGM, MUC6 (CLH5, Novocastra Lab), CD10 (56C6, Novocastra Lab), or CDX2 (CDX-2-88, Biogenex, San-Roman, CA, USA). Then, the sections were subjected to the Envision kit (Dako, Glostrup, Denmark) including the secondary antibodies. Immunoreacted cells were visualized with diaminobenzidine tetrahydrochloride, and nuclei were lightly counterstained with hematoxylin. Positive controls comprised normal pancreatic duct cells for MUC1/DF3 and MUC1/CORE, mature goblet cells of normal colonic mucosa for MUC2, normal gastric mucosa for MUC5AC/CLH2 and HGM, normal pyloric gland for MUC6, normal intestinal mucosa for CDX2, and normal small intestinal mucosa for CD10.

Scorings of immunohistochemical results

Immunohistochemical stains were graded by the presence of positively stained tumor cells as follow: -, less than 5% of tumor cells: +, 5% to 50% of tumor cells: and ++, over 50% of tumor cells. The cases showing + and ++ were evaluated as "positive".

Statistical analysis

The value was shown as mean ± SE. Statistical analysis was carried out by the Student's *t*-test or χ^2 test (Excel: Microsoft, Redmond, WA, USA). A *P*-value below 0.05 was considered significant.

RESULTS

Clinical and immunohistochemical characteristics of each case are shown in Table 1. There was no significant difference between patients with low-grade MAC and those with high-grade MAC in both sex ratio (M:F = 2:4 *vs* 4:6) and age distribution (67.8 ± 7.53 *vs* 58.6 ± 3.53, $P = 0.229$ at *t*-test). MUC1/DF3 and MUC1/CORE were immunolocalized on the membrane and/or

Table 1 Clinical and immunohistochemical characteristics

Case	Location	Sex	Age	Grade	MUC1/DF3	MUC1/CORE	MUC2	MUC5AC/CHL2	HGM	MUC6	CD10	CDX2
1	T	F	71	Low	-	-	++	+	+	-	-	-
2	R	M	71	Low	-	-	++	++	+	-	-	++
3	A	F	83	Low	+	-	++	++	++	-	-	++
4	A	F	90	Low	+	+	++	++	+	-	-	++
5	R	F	48	Low	+	-	++	+	++	-	-	++
6	R	M	44	Low	-	-	++	+	-	-	-	++
7	R	F	75	High	++	++	++	+	+	-	-	-
8	T	F	57	High	++	+	++	-	+	-	-	++
9	R	M	47	High	++	+	+	-	-	-	-	+
10	R	M	63	High	+	-	++	+	+	-	-	++
11	A	M	57	High	+	-	++	-	-	-	-	-
12	A	F	68	High	+	+	++	-	-	-	-	++
13	T	F	48	High	++	+	+	+	++	-	-	+
14	R	F	75	High	++	+	++	-	+	-	-	-
15	R	M	48	High	++	+	+	-	-	-	-	+
16	A	F	48	High	+	-	++	+	+	-	-	++

T: Transverse colon; R: Rectum; A: Ascending colon; F: Female; M: Male. Immunohistochemical stains were graded by the presence of positively stained tumor cells as follow: -, less than 5% of tumor cells; +, 5% to 50% of tumor cells; and ++, over 50% of tumor cells.

Table 2 Summary of immunostaining

Antibodies	Low-grade MAC (%)	High-grade MAC (%)
MUC1/DF3[1]	3/6 (50)	10/10 (100)
MUC1/CORE	1/6 (16.7)	7/10 (70)
MUC2	6/6 (100)	10/10 (100)
MUC5AC/CHL2[1]	6/6 (100)	4/10 (40)
HGM	5/6 (83.3)	6/10 (60)
MUC6	0/6 (0)	0/10 (0)
CD10	0/6 (0)	0/10 (0)
CDX2	5/6 (83.3)	7/10 (70)

Proportions (%) of cases evaluated as "positive" are shown. Statistical analysis between low- and high-grade MAC was carried out by χ^2 test. [1]A *P*-value below 0.05 was considered significant. MAC: Mucinous adenocarcinoma.

intracytoplasmic lumen of tumor cells. MUC1/DF3 was positive in 3 of 6 low-grade MAC (50%) and 10 of 10 high-grade MAC (100%). MUC1/CORE was positive in 1 of 6 low-grade MAC (16.7%) and 7 of 10 high-grade MAC (70%). MUC2 was immunolocalized in the cytoplasm of tumor cells. MUC2 was expressed in all MAC regardless of the grade. MUC5AC/CHL2 and HGM were immunolocalized in the cytoplasm of tumor cells with goblet or columnar cell features. MUC5AC/CHL2 was positive in 6 of 6 low-grade MAC (100%) and 4 of 10 high-grade MAC (40%). HGM was positive in 5 of 6 low-grade MAC (83.3%) and 6 of 10 high-grade MAC (60%). MUC6 and CD10 were not detected in any MAC regardless of the grade. CDX2 was immunolocalized in the nucleus of tumor cells. CDX2 was positive in 5 of 6 low-grade MAC (83.3%) and 7 of 10 high-grade MAC (70%). Taken together, MUC1/DF3 was expressed significantly more frequently in high-grade MAC than in low-grade ($P = 0.131$, χ^2 test), and MUC5AC/CHL2 was expressed significantly more frequently in low-grade MAC than in high-grade ($P = 0.164$, χ^2 test) (Table 2). Representative immunostaining patterns of MUC1/DF3, MUC2, and MUC5AC/CLH2 in low- and high-grade MAC are shown in Figure 1.

DISCUSSION

It has been reported that MUC1 is frequently expressed in invasive carcinomas, but not non-invasive carcinomas in various tissues, suggesting that the expression of MUC1 is related to increasing tendency for malignancy and invasion[13-15]. In the non-specific conventional adenocarcinoma of colon and rectum, MUC1 is considered to be a prognostic marker and served as a biological feature associated with the aggressiveness of advanced carcinomas[7]. We report that MUC1/DF3 was expressed significantly more frequently in high-grade MAC than in low-grade MAC in the colon and rectum. These results support that MUC1 is one of the indices of malignancy of tumors, and suggest that MUC1/DF3 immunostaining is useful to distinguish between low- and high-grade MAC.

MUC5AC is expressed in adenoma and conventional adenocarcinoma with well to moderate differentiation in the colon and rectum[16-20]. In addition, MUC5AC has also been reported to be expressed in 56%-63% cases of colorectal MAC[17,18]. On the other hand, Kocer *et al*[19] have reported that the absence of MUC5AC expression in tumors can be a prognostic factor for more aggressive adenocarcinoma in the colon and rectum. In this study, we found that the frequency of MUC5AC/CHL2 expression was significantly lower in high-grade MAC compared with low-grade MAC. These findings are consistent with the reports by Kocer *et al*[19], and indicate that decreases in MUC5AC expression are a prognostic marker for aggressive and advanced MAC.

MUC2 has been reported to be expressed in poorly differentiated adenocarcinoma in the colon and rectum[16-18]. In this study, MUC2 was expressed in all colorectal MAC regardless of the grade. These results indicate that MUC2 is a positive marker for colorectal MAC but is not suitable to distinguish between low-grade MAC and high-grade MAC. MUC6 and CD10 were not detected in any MAC regardless of the grade. These molecules may be negative

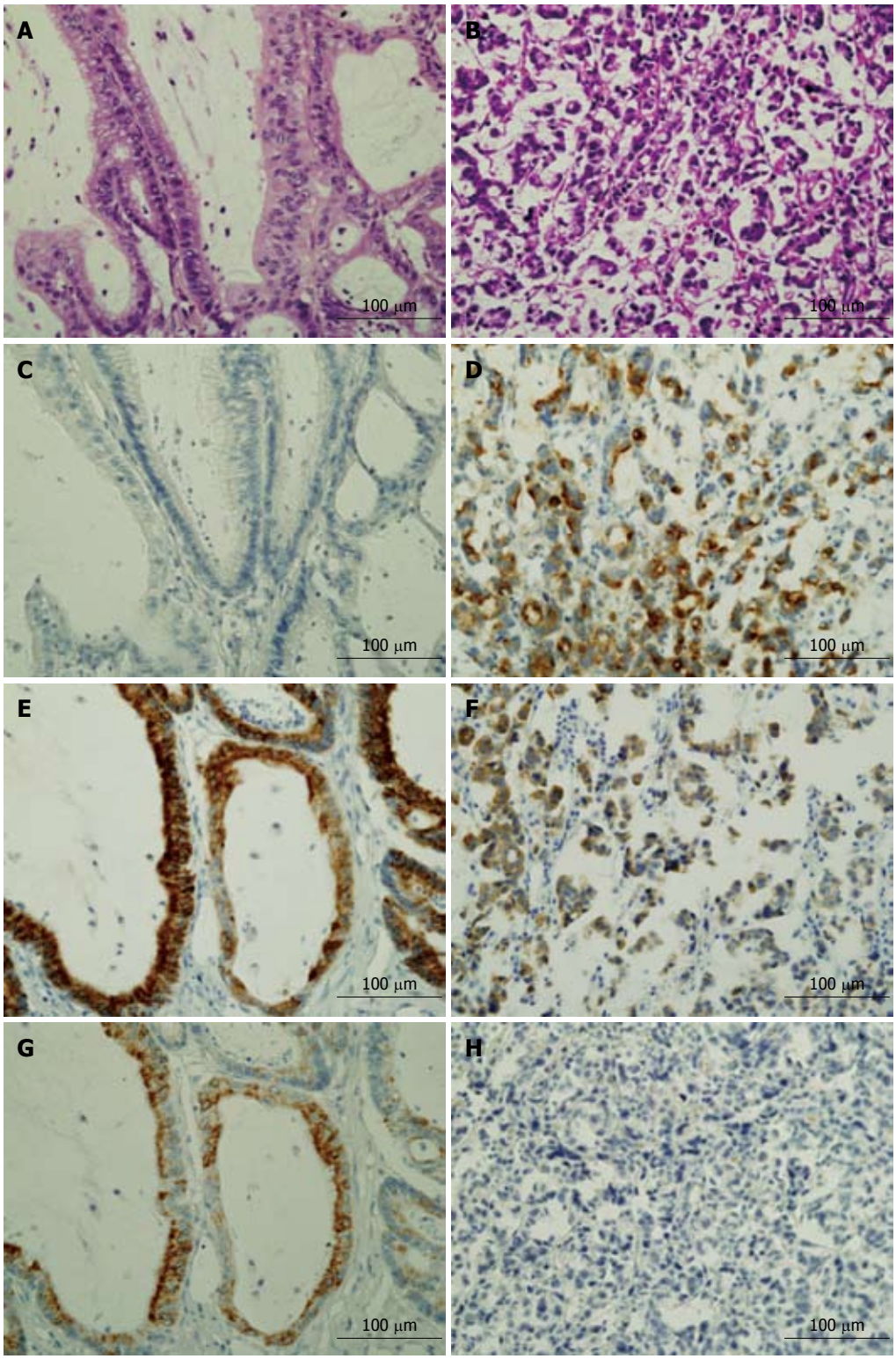

Figure 1 Histological characteristics of MAC. A, C, E, and G show histological characteristics of case #1 (low-grade MAC) and B, D, F and H those of case #8 (high-grade MAC). C and D: Immunostained with MUC1/DF3. E and F: Immunostained with MUC2; G and H: Immunostained with MUC5AC/CHL2.

markers for colorectal MAC.

In summary, we compared immunohistochemical expression of MUC1, MUC2, MUC5AC, MUC6, CD10 and CDX2 between low- and high-grade MAC, and found that increased MUC1 and decreased MUC5AC expressions are related to malignant potential of colorectal MAC. Since the expression of MUC1/DF3 and MUC5AC/CHL2 differed significantly between low- and high-grade MAC in the colon and rectum, it is proposed that MUC1/DF3 and MUC5AC/CHL2 immunostaining is useful to distinguish between these two types of MAC.

COMMENTS

Background

Mucinous adenocarcinoma (MAC) is characterized by pools of extracellular mucin that contain malignant epithelium. MAC can be divided into two types based on the degree of structural differentiation; low-grade MAC arisen from well to moderately differentiated adenocarcinoma and papillary carcinoma, and high-grade MAC arisen from poorly differentiated adenocarcinoma and signet ring cell carcinoma. However, useful markers for the differential diagnosis of low- and high-grade MAC in the colon and rectum have not been identified.

Innovations and breakthroughs

The immunohistochemical expression of 2 types of MUC1 (MUC1/DF and MUC1/CORE), MUC2, 2 types of MUC5AC (MUC5AC/CHL2 and HGM), MUC6, CDX2, and CD10 was compared between low- and high-grade MAC. MUC1/DF3 was expressed significantly more frequently in high-grade MAC than in low-grade, and MUC5AC/CHL2 was expressed significantly more frequently in low-grade MAC than in high-grade. These results indicate that increased MUC1 and decrease MUC5AC expressions are related to malignant potential of colorectal MAC.

Applications

It is proposed that MUC1/DF3 and MUC5AC/CHL2 immunostaining is useful to discriminate high-grade MAC from low-grade MAC in the colon and rectum.

Terminology

MAC is defined as a carcinoma with mucin composing more than 50% of the lesion. Mucin, a high molecular weight glycoprotein, is the major component in the mucus gel on epithelial surfaces with a characteristic organ- and cell type-specific distribution. Mucin binds to pathogens as part of the immune system.

Peer review

This is a good descriptive study in which authors compared the expression of mucins (MUC1, MUC2, MUC5AC, and MUC6), CD10, and CDX2 between low- and high-grade MAC. The results are interesting and indicate that MUC1 and MUC5AC are useful markers to discriminate high-grade MAC from low-grade MAC in the colon and rectum. The combination of markers in this study is quite unique.

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BRIEF ARTICLE

Selenium as a chemopreventive agent in experimentally induced colon carcinogenesis

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Abstract

AIM: To elucidate the chemopreventive efficacy of selenium during experimentally induced colon carcinogenesis.

METHODS: Thirty-two male wistar rats were divided into four groups: group Ⅰ (normal control); group Ⅱ [1,2-dimethylhydrazine (DMH) treated]; group Ⅲ (selenium treated); and group Ⅳ (DMH + selenium treated). Groups Ⅱ and Ⅳ were given subcutaneous injections of DMH (30 mg/kg body weight) every week for 20 wk. Selenium, in the form of sodium selenite, was given to groups Ⅲ and Ⅳ at 1 ppm in drinking water ad libitum for 20 wk. At the end of the study, rats were sacrificed and their colons were analyzed for the development of tumors, antioxidant enzyme levels and histological changes.

RESULTS: 100% of the DMH treated rats developed tumors, which was reduced to 60% upon simultaneous selenium supplementation. Similarly, tumor multiplicity decreased to 1.1 following selenium supplementation to DMH treated rats. Levels of lipid peroxidation, glutathione-S-transferase, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) decreased following DMH treatment, whereas levels of glutathione (GSH) and glutathione reductase (GR) significantly increased in DMH treated rats. Selenium administration to DMH treated rats led to an increase in the levels of lipid peroxidation, SOD, catalase, glutathione-S-transferase and GPx, but decreased the levels of GSH and GR. Histopathological studies on DMH treated rats revealed dysplasia of the colonic histoarchitecture, which showed signs of improvement following selenium treatment.

CONCLUSION: The study suggests the antioxidative potential of selenium is a major factor in providing protection from development of experimentally induced colon carcinogenesis.

Key words: Colon cancer; Selenium; Antioxidant enzyme; Histopathology; Dimethylhydrazine

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INTRODUCTION

Colorectal cancer is amongst the leading causes of cancer-related deaths and one of the most commonly diagnosed cancers[1]. The oxidation of biomolecules due to reactive oxygen species (ROS) is associated with cellular dysfunction and leads to various biological responses, such as inflammation and apoptosis. When ROS attack DNA, oxidized bases are generated and the unrepaired oxidative DNA damage can induce mutations. Formation of hydroxylated bases of DNA is considered as an important event in chemical carcinogenesis[2,3]. Colon carcinogenesis is a multistep process in which oxygen radicals were found to enhance carcinogenesis at all stages: initiation, promotion, and progression[4]. The colon carcinogen 1,2-dimethylhydrazine (DMH) has been widely used to chemically induce colon cancers[5].

Dietary constituents have been reported to play vital roles in the development or prevention of cancer. Selenium, an essential trace nutrient, has been reported to improve immune function in animals[6,7], enhance neuropsychological function in humans[8] and ameliorate specific disease conditions in humans and animals[9]. The relationship between selenium and the etiology of cancer in humans remains elusive and intriguing, despite the number of studies published on the topic.

Selenium deficiency has been associated with initiation of events leading to the development of tumors[10,11]. Low levels of selenium have been associated with a higher risk of cardiovascular diseases and cancer in humans, which is another important factor related to dietary intake[12]. Epidemiological studies illustrate an increased incidence of colorectal cancer in humans in geographic regions where selenium is deficient[13]. Selenium affects colon cancer susceptibility and DNA methylation. Animals fed selenium-deficient diets had significantly hypomethylated colonic DNA compared with those fed diets supplemented with selenite or selenomethionine[14]. Thus, alterations in DNA methylation might help explain the increased tumorigenesis associated with selenium deficiency.

The present study was carried out to further explore the chemopreventive efficacy of selenium, if any, on the initiation and progression of colon cancer induced with DMH in a rat model.

MATERIALS AND METHODS

Chemicals

DMH, reduced nicotinamid adenine dinucleotide, glutathione (GSH), nitroblue tetrazolium, 5,5'-dithiobis 2-nitrobenzoic acid, were procured from Sigma-Aldrich (Delhi, India). Sodium selenite was purchased from E. Merck. All the chemicals used were of analytical grade.

Animals

Male wistar rats, in the weight range 120-150 g, were procured from the central animal house, Panjab University, Chandigarh. All the animals were housed in polypropylene cages under hygienic conditions. Basal supplemented diets (Ashirwad Industries, Punjab, India) were given to the animals. Before initiating the experiments, the animals were adapted to the laboratory conditions for a week. All the procedures were performed in accordance with the standard guidelines for care and use of laboratory animals and the protocols followed were approved by the Institute's Ethical Committee on animals.

Experimental design

Thirty-two animals were randomly and equally assigned into four treatment groups. Animals in Group I served as normal controls and were given water and diet *ad libitum*. Rats in this group were also administered with 1 mmol/L EDTA-saline subcutaneously per week, which was used as a vehicle for the DMH treatments. Animals in Group Ⅱ were given subcutaneous injections of DMH [dissolved in 1 mmol/L EDTA-normal saline (pH 6.5)] every week at 30 mg/kg body weight for 20 wk[15]. Group Ⅲ animals were given selenium in the form of sodium selenite in drinking water *ad libitum* at 1 ppm in drinking water. Animals in Group Ⅳ were given a combined treatment of DMH as well as selenium, similarly to Group Ⅱ and Group Ⅲ animals, respectively.

Record of body weights

A record of the body weights of normal control, DMH and selenium treated animals was kept throughout the study. The animals were weighed at the beginning of the experiment, once a week during the experiment and finally before sacrifice.

Colon tumor analysis

After 20 wk of DMH treatment, colons were excised from the rats, blotted dry, opened longitudinally and the inner surface was examined for visible macroscopic lesions. Tumors were easily discernable in the inflamed sections of the colon. The colons were observed for tumor incidence and multiplicity studies. Tumor size was recorded using a vernier caliper with 0.1 mm graduations. The chemopreventive tumor response was assessed on the basis of tumor incidence and multiplicity, which were calculated as follows: Tumor incidence, percentage of animals having tumors; Tumor multiplicity, mean of tumors counted/animals.

Preparation of colon homogenates

Animals from all the groups were sacrificed by cervical dislocation under light ether anesthesia at the end of the study. Their colons were removed and washed with ice chilled saline. Colon homogenates (10%) were prepared in ice cold Tris-Mannitol buffer (2 mmol/L Tris, 50 mmol/L Mannitol, pH 7.2) using a mechanically driven Teflon fitted Potter-Elvehjem type homogenizer for a few minutes to achieve total disruption of cells. Homogenates were centrifuged at 10 000 *g* for 10 min at 4℃. Aliquots of the supernatants were prepared and stored at -20℃ for various biochemical investigations.

Table 1 Effect of selenium on body weights of animals subjected to 20 wk of DMH treatment

Groups	Body weight (g)	
	0 d	20 wk
Ⅰ normal control	135 ± 15.00	282 ± 16.43
Ⅱ DMH	134 ± 16.73	210 ± 15.81[d]
Ⅲ selenium	134 ± 15.11	284 ± 37.81
Ⅳ DMH + selenium	134 ± 20.70	246 ± 33.61[a,b]

[b]$P < 0.01$ and [d]$P < 0.001$ by one-way ANOVA followed by LSD test when values are compared with normal control group; [a]$P < 0.05$ by one-way ANOVA followed by LSD test when values of group Ⅳ animals are compared with group Ⅱ animals. Values are expressed as mean ± SD.

Lipid peroxidation and antioxidant defense system enzymes

Lipid peroxidation was assayed according to the method of Wills[16]. One of the end products of lipid peroxidation is malondialdehyde (MDA), which forms a pink colored complex with thiobarbituric acid with an absorption maxima at 532 nm. Glutathione-Peroxidase enzyme activity was assayed using glutathione reductase and H_2O_2 as substrates, and the optical density was read at 340 nm with a double beam spectrophotometer[17]. The activity of total SOD was measured at 560 nm following the method of Kono[18]. The enzymatic determination of catalase was performed according to the method of Luck[19] and the concentration of H_2O_2 was monitored at 240 nm. The activity of glutathione-S-transferase was estimated according to the method of Habig *et al*[20]. Reduced GSH contents were determined using the method of Ellman[21]. Glutathione reductase (GR) activity was assayed using the method of Carlberg *et al*[22].

Histopathological studies

Formalin fixed tissues were processed for histopathological observations at the light microscopic level. Briefly, following an overnight fixation in buffered formalin, tissues were dehydrated through ascending grades of alcohol, cleared in benzene and embedded in paraffin. Sections of 5-7 micrometer thick were cut, placed serially on clean glass slides and then de-paraffinized through descending grades of alcohol. Sections were made from each colon tissue and were stained with hematoxylin and eosin. These were then observed under a light microscope and the gross morphology was noted.

Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and a multiple post hoc test (LSD). The significance was set at $P < 0.05$. The results are represented as mean ± SD.

RESULTS

Body weight changes

The variations in the body weights of the animals subjected to different treatments are shown in Table 1. The body weights of all the normal and treated animals (Table 1) rose steadily throughout the study. However, the body weight gains of the animals treated with DMH was markedly less as compared to the normal controls. Selenium treatment of DMH treated rats tended to improve the body weight growth in comparison to DMH treated animals.

Table 2 Chemopreventive efficacy of selenium on the tumor incidence, tumor multiplicity and tumor size of DMH-induced rat colonic tumors

Groups	Colon tumor incidence (percentage of tumor bearing rats)	Colon tumor multiplicity (mean tumor/animal)	Tumor size (cm)
Normal control	0	0	-
DMH	100%	2.6	0.911 ± 0.196
Selenium	0	0	-
DMH + selenium	60%	1.1	0.609 ± 0.250[b]

[b]$P < 0.001$ by one way-ANOVA followed by LSD test when values of Ⅳ animals were compared with Group Ⅱ animals. Values are expressed as mean + SD.

Colon tumor analyses

Tumor incidence was observed to be 100% in DMH group of rats. Further, the tumor incidence was reduced in the DMH treated rats that were supplemented with selenium (Table 2). Similarly, tumor multiplicity that increased following DMH treatment, tended to decrease upon selenium supplementation. In addition, a significant reduction in colon tumor size was also evident in Group Ⅳ rats when compared to DMH treated rats.

Antioxidant defense system enzymes and lipid peroxidation

In the present study, MDA levels as a direct indicator of lipid peroxidation, were decreased significantly ($P < 0.001$) after 20 wk of DMH treatment. Selenium treatment to normal rats did not indicate any significant change in MDA levels. Selenium supplementation to DMH treated rats significantly reversed ($P < 0.01$) the otherwise altered levels of LPO observed at the end of the study.

DMH treatment to normal animals resulted in a significant decrease in the enzymes activities of GST, SOD, catalase and GPx ($P < 0.001$). In contrast, a significant increase ($P < 0.01$) in the levels of GSH and the enzyme activity of GR was observed following DMH treatment (Table 3). However, selenium treatment to DMH treated animals resulted in a significant elevation in the activities of enzymes GST, catalase, GPx and SOD, but caused a significant decrease in the levels of GSH and GR when compared with DMH treated group.

Histopathology

Histopathological analysis showed that the colon of rats from normal and selenium treated groups had normal histoarchitecture with no signs of apparent abnormality (Figure 1A and C). In the DMH treated groups, well differentiated signs of dysplasia were observed. Nuclei

Table 3 Effect of selenium on lipid peroxidation and antioxidant enzymes in the colons of rats subjected to 20 wk of DMH treatment

Groups	Lipid peroxidation (nmoles of MDA/ min/100 mg protein)	GR (mmol NADPH oxidized/ min/mg protein)	GST (μmol of conjugate formed/ min/mg protein)	SOD (I.U)	CAT (mmol of H_2O_2 decom posed/ min/mg protein)	GSH (μmol GSH/g tissue)	GPx (mmol NADPH oxidized/ min/mg protein)
Normal control	3.229 ± 0.38	1.375 ± 0.066	0.158 ± 0.073	6.430 ± 0.617	1.234 ± 0.200	0.629 ± 0.011	0.706 ± 0.02
DMH	2.237 ± 0.35[d]	1.708 ± 0.101[d]	0.033 ± 0.021[d]	4.438 ± 0.316[d]	0.794 ± 0.043[d]	1.089 ± 0.023[d]	0.434 ± 0.15[d]
Selenium	3.239 ± 0.20	1.334 ± 0.217	0.137 ± 0.027	6.287 ± 0.524	1.230 ± 0.113	0.637 ± 0.017	0.711 ± 0.015
DMH + selenium	3.032 ± 0.21[f]	1.546 ± 0.043[a,c]	0.069 ± 0.022[b]	5.695 ± 0.417[a,f]	1.029 ± 0.278[c]	0.646 ± 0.027[f]	0.614 ± 0.17[d,f]

[a]$P < 0.05$, [b]$P < 0.01$ and [d]$P < 0.001$ by one way-ANOVA followed by LSD test when values are compared with control group; [c]$P < 0.05$ and [f]$P < 0.001$ by one way-ANOVA followed by LSD test when values of Group Ⅳ animals are compared with Group Ⅱ animals. Values are expressed as mean + SD.

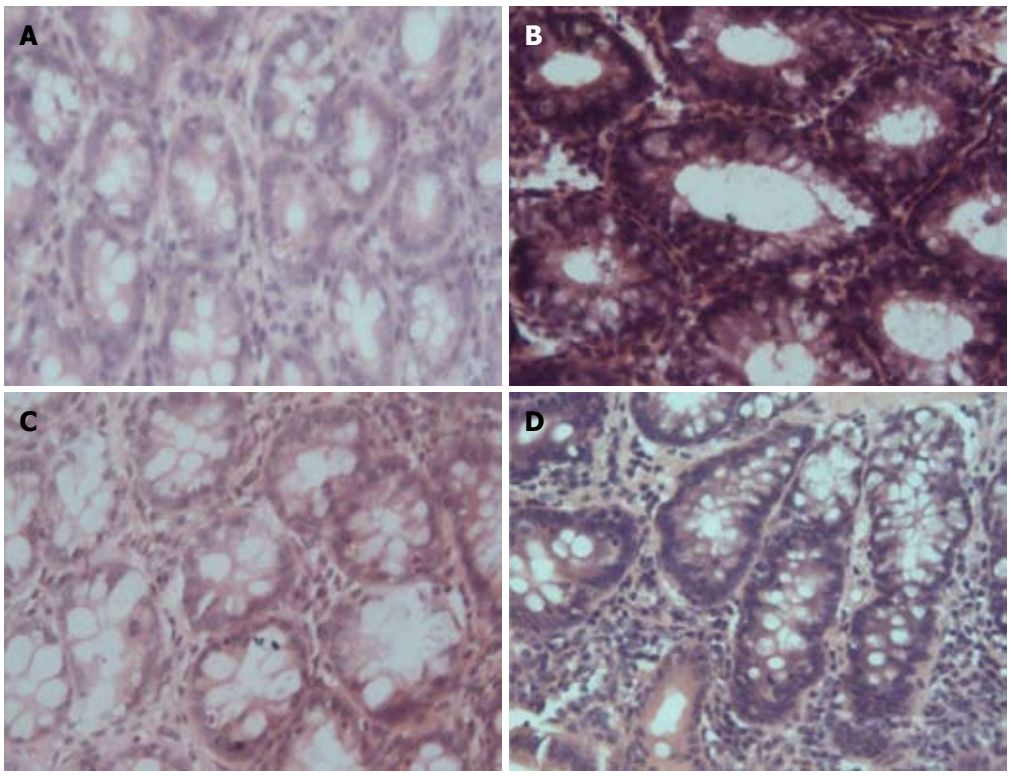

Figure 1 Histo-micrograph of rat colon. A: Normal histoarchitecture of rat colon (× 40); B: Altered colonic histoarchitecture from DMH treated rats (× 40); C: Colonic histoarchitecture from selenium treated rats (× 40); D: Colonic histoarchitecture from DMH + selenium treated rats (× 40).

were enlarged, thickening of epithelium was seen, cells were hyper-chromatic and showed increased mitotic activity. Simultaneously, there was a loss in nuclear polarity (Figure 1B). In the combined treatment group (Group Ⅳ), the histoarchitecture revealed no signs of dysplasia, but did indicate some loss of nuclear polarity (Figure 1D).

DISCUSSION

The protective effects of selenium on the histoarchitecture and oxidative stress enzymes were observed in an experimental model of DMH-induced colon carcinogenesis. The study clearly indicates that the administration of selenium attenuates the DMH induced alterations in the levels of lipid peroxidation and the overall antioxidant enzymatic status in the rat colons. Furthermore, the histological findings clearly support these biochemical data and suggest that selenium might play a promising anticancer role with respect to colon carcinogenesis. In this context, other chemopreventive agents with antioxidant properties have been found to inhibit DMH and azoxymethane-induced colon carcinogenesis and DNA damage in an animal model[23].

In the present study, selenium treatment to DMH treated rats for 20 wk caused a reduction in tumor incidence and tumor multiplicity, with a concomitant reduction in average tumor size. These data strongly suggest that selenium has the potential to inhibit/slow tumorigenesis in the rat colon. Moreover, the absence of tumor incidence in rats treated with selenium alone suggests that selenium, at this dose level, causes no disruption of normal cellular homeostasis and hence is non-toxic.

The levels of lipid peroxidation in the colon tissues were decreased after 20 wk of DMH treatment. Previous studies have shown reduced rates of lipid peroxidation in the tumor tissue of various types of cancers[24-27]. It has been claimed that MDA acts as a tumor promoter and

co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes[28]. There are contradictory results on this subject in the literature with regard to cancerous conditions. Several investigators also reported that MDA levels were significantly increased in cancerous tissue when compared to healthy controls[29,30]. Devasena *et al*[31] reported increased, tumor incidence as well as enhanced LPO, in the circulation of colon tumor bearing rats. On the other hand, Gerber *et al*[32] reported that MDA levels decreased with increasing tumor size and progression in breast cancer. Certain studies have reported an inverse relationship between lipid peroxidation and cell proliferation[33]. Our results indicate that a decrease in the levels of MDA can be attributed to increased cell proliferation, which is thought to be involved in the pathogenesis of colon cancer. Cancer cells acquire particular characteristics that promote their proliferation[34] and tend to proliferate faster when the lipid peroxidation level is low. Therefore, the decreased lipid peroxidation observed in DMH-treated rats could be due to increased cell proliferation. The malignant tissues seem to be less susceptible and more resistant to free radical attack, and hence lipid peroxidation is less intense[35]. Interestingly, simultaneous selenium treatment to DMH treated animals showed an increase in the levels of MDA.The observed increased levels of LPO under selenium treatment could be as a consequence of the inhibitory action of selenium on the proliferative activity of cancerous cells. We also observed reduced catalase levels in DMH treated rats. The fall in catalase activity correlated well with tumor stage according to Dukes, suggesting that this peroxisomal enzyme could be used as a potential prognostic marker[36]. Decreased lipid peroxidation associated with enhanced GSH in the colon and intestines is a well known phenomenon in experimental carcinogenesis[37]. We have also observed enhanced GSH levels following 20 wk of DMH treatment. This might be due to the increased cell proliferation involved in the pathogenesis of DMH-induced colon cancer[38]. It was previously demonstrated that GSH is expressed in greater amounts in the neoplastic cells, conferring a selective growth advantage[39]. It has also been reported that DMH treatment results in increased tissue GSH content[40]. In the presence of GSH as a substrate and GPx and GST as detoxifying enzymes, conjugation of toxic electrophiles with GSH takes place, conferring a selective growth advantage to cancer cells. Thus, the elevated GSH levels in the colon, as observed in our study might be used as a marker of cell proliferation. Interestingly, treatment with selenium to DMH treated animals modulated the levels of GSH, thus ascribing their protective effect in restoring GSH activity. Furthermore, the results of increased GSH levels are in accordance with the findings of increased levels of glutathione reductase and decreased levels of glutathione-S-transferase. The antioxidant enzymes SOD, GPx and catalase limit the effects of oxidant molecules on tissues and are activated in the defense against oxidative cell injury by means of their being free radical scavengers[41]. These enzymes work together to eliminate active oxygen species and small deviations in physiological concentrations may have a dramatic effect on the resistance of cellular lipids, proteins and DNA to oxidative damage[42]. In the present study, SOD, GPx and catalase activities were found to be significantly decreased following 20 wk DMH treatment, when compared to the normal control animals. The decreased enzyme activities of SOD, GPx and catalase could be due to post-translational or oxidative modification of ROS scavenging enzymes[43]. The protective effect observed upon supplementation of selenium indicates that selenium eliminates the toxic effects of DMH on the activity of these enzymes.

Selenium is an essential component of several enzymes such as glutathione peroxidase (GSH-Px), thioredoxin reductase (TR) and selenoprotein P (SeP), which contains selenium as selenocysteine. Selenium is also essential for cell culture when a serum-free medium is used[44,45]. The antioxidant activity of selenium can be explained by its important role in preventing lipid peroxidation and in protection of integrity and functioning of tissues and cells. The role of selenium in preventing lipid peroxidation and oxidative damage has also been demonstrated in colon studies[44,46]. Moreover, Saito *et al*[45] have reported that selenium and Vit E show compensative effects and that a deficiency of both elements might cause massive injury. Selenium compounds are known for their antioxidative ability, therefore another favorable explanation is that selenium compounds affect carcinogen activation and metabolism through inhibition of phase I enzymes and induction of phase Ⅱ enzymes[47-49].This mechanism has been well documented to be important for the chemo-preventive activity of many thiol-reactive chemopreventive agents[50-53].

The ability of selenium compounds to inhibit growth and induce tumor cell apoptosis has been suggested to be a potential mechanism for cancer chemoprevention[47]. Amagase *et al*[54] and Ip *et al*[55] reported that selenium, supplied either as a component of the diet or as a constituent of a garlic supplement, enhanced protection against 7,12-di methylbenz[*a*]anthracene induced mammary carcinogenesis over that provided by garlic alone. Suppression in carcinogen bioactivation, as indicated by a reduction in DNA adducts, might account in part for this combined benefit of garlic and selenium[56].

The histopathological observations suggest that supplementation of selenium under the experimental conditions can greatly affect the post-initiation stages of colon carcinogenesis by altering the efficacy at which DMH can initiate histological changes. Well-differentiated signs of dysplasia were observed in colonic tissue sections by DMH administration alone. Treatment with selenium greatly restored the normal histoarchitecture in the colonic epithelial cells, with no apparent signs of dysplasia. The ability of selenium to restore the histological changes induced by DMH indicates the anti-carcinogenic potential of this trace metal. Increased antioxidant defense upon selenium

treatment could lower the ROS-mediated damage at the initiation as well as during progression/promotion phase of tumorigenesis. The antioxidative activity of organoselenium compounds is believed to be based on the prominent role that selenium plays in many of the enzymes of the oxidative defense system[57-61]. Studies have demonstrated that selenium supplementation reduces the incidence of cancer, particularly prostate cancer[62,63]. Evidence from experimental studies suggests that apoptosis is a key event in cancer chemoprevention by selenium and reactive oxygen species play a role in induction of apoptosis by selenium compounds. Xiang *et al*[63] found that selenite induces cell death and apoptosis by production of superoxide in mitochondria and activation of the mitochondrial apoptotic pathway and MnSOD plays an important role in protection against pro-oxidant effects of superoxide from selenite during proliferative phase. The data suggest that superoxide production in mitochondria is, at least in part, a key event in selenium-induced apoptosis in prostate cancer cells. Ganther[48] reported that the metabolism of selenium compounds is a prerequisite for cancer prevention. Extensive studies have concluded that selenium compounds directly converted to mono-methylated forms, (methylselenol, CH3SeH) or related intermediates (e.g. aromatic selenol) are powerful chemopreventive agents. The possible mechanisms by which selenium is postulated to decrease the incidence of cancer include inhibition of oxidative damage to DNA, recharging of cellular proliferation, modulation of apoptosis, and alteration of cellular gene expression.

In conclusion, the results of this study suggest that selenium has a positive beneficial effect against the chemically induced colonic preneoplastic progression in rats induced by DMH, which provides an effective dietary chemopreventive approach to manage the disease. However, further studies are warranted with regard to other bioassays, including protein expression and documentation of specific molecular markers to establish the exact mechanism for selenium-mediated chemoprevention of cancer.

COMMENTS

Background

The process of carcinogenicity presents a major challenge to scientists. Cancer chemoprevention refers to the use of pharmacological agents to inhibit, delay or reverse the multi-step process of carcinogenesis. The last two decades in particular have witnessed explosive growth in this emerging field of cancer chemoprevention. Many classes of agents include antioxidants and other diets have shown promise as chemopreventive agents. Therefore, for reducing the incidence of cancer, modifications in dietary habits, especially by increasing consumption of fruits and vegetables rich in antioxidants are increasingly advocated. However, in the present study, selenium has been proposed as chemopreventive agent to reduce the incidence of cancer in an animal model. Biochemical and histological techniques used to detect the changes in the antioxidant activity and microscopic alterations in the colon tissues of the animals has been reported.

Research frontiers

The alteration of antioxidant enzymes activity can be analyzed by biochemical estimation and histopathological study when colon cancer takes place because of the toxicity of pro-carcinogen 1,2-dimethylhydrazine (DMH). The results of present article will be helpful for carrying out further studies concerning chemopreventive role of selenium.

Innovations and breakthroughs

In the present study, results from both biochemical assays and histological study showed reactive oxygen species increase in the rats due to pro-carcinogen DMH. Selenium supplementation to the DMH treated animals changed the altered levels of antioxidant enzymes due to its important antioxidative property.

Applications

This study is useful to explain the anti-oxidative/anti-tumor activity of selenium. It might also play an important role in the treatment of tumors.

Peer review

It is widely known that selenium can inhibit colon carcinogenesis induced by repeated injection of DMH, but the precise mechanisms remain to be investigated. The authors provided evidence to indicate that selenium can exert anti-oxidant activities and eventually protect this colon carcinogenesis. The observations are novel and merit publication.

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BRIEF ARTICLE

Studies on activity of various extracts of *Mentha arvensis* Linn against drug induced gastric ulcer in mammals

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Abstract

AIM: To examine the antiulcerogenic effects of various extracts of *Mentha arvensis* Linn on acid, ethanol and pylorus ligated ulcer models in rats and mice.

METHODS: Various crude extracts of petroleum ether, chloroform, or aqueous at a dose of 2 g/kg po did not produce any signs or symptoms of toxicity in treated animals. In the pyloric ligation model oral administration of different extracts such as petroleum ether, chloroform and aqueous at 375 mg/kg *po*, standard drug ranitidine 60 mg/kg *po* and control group 1% Tween 80, 5 mL/kg *po* to separate groups of Wister rats of either sex (n = 6) was performed. Total acidity, ulcer number, scoring, incidence, area, and ulcer index were assessed.

RESULTS: There was a decrease in gastric secretion and ulcer index among the treated groups i.e. petroleum ether (53.4%), chloroform (59.2%), aqueous (67.0%) and in standard drug (68.7%) when compared to the negative control. In the 0.6 mol/L HCl induced ulcer model in rats (n = 6) there was a reduction in ulcerative score in animals receiving petroleum ether (50.5%), chloroform (57.4%), aqueous (67.5%) and standard. drug (71.2%) when compared to the negative control. In the case of the 90% ethanol-induced ulceration model (n = 6) in mice, there was a decrease in ulcer score in test groups of petroleum ether (53.11%), chloroform (62.9%), aqueous (65.4%) and standard drug ranitidine (69.7%) when compared to the negative control. It was found that pre-treatment with various extracts of *Mentha arvensis* Linn in three rat/mice ulcer models ie ibuprofen plus pyloric ligation, 0.6 mol/L HCl and 90% ethanol produced significant action against acid secretion (49.3 ± 0.49 *vs* 12.0 ± 0.57, $P < 0.001$). Pre-treatment with various extracts of *Mentha arvensis* Linn showed highly -significant activity against gastric ulcers (37.1 ± 0.87 *vs* 12.0 ± 0.57, $P < 0.001$).

CONCLUSION: Various extracts of *Mentha arvensis* Linn. 375 mg/kg body weight clearly shows a protective effect against acid secretion and gastric ulcers in ibuprofen plus pyloric ligation, 0.6 mol/L HCl induced and 90% ethanol-induced ulcer models.

Key words: *Mentha arvensis*; Anti ulcer; Gastro-protection; Medicinal plant

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INTRODUCTION

Ulcers are a crater-like erosion or sore that occur in the upper gastrointestinal tract of the body. Stomach ulcers are also called peptic ulcers[1]. The word peptic refers to pepsin, a stomach enzyme that breaks down protein. A peptic ulcer located in the stomach is called a gastric ulcer. An ulcer is the result of an imbalance between aggressive and defensive factors. On one hand, too much acid and pepsin can damage the stomach lining and cause ulceration. On the other hand, the damage comes first from some other cause making the stomach lining susceptible to even an ordinary level of gastric acid[2]. Peptic ulceration is a very common disease and it is estimated that approximately 10%-20% of the adult male population in western countries will experience a peptic ulcer at some stage in their lives[3]. It produces considerable pain and illness. In 1970 in the USA 3.5 million people suffered from peptic ulcer and 8600 deaths were attributed to this disease. At present nearly 15 million people are suffering from peptic ulcer diseases and 6000 deaths per year are reported across the world. According to physicians more than 90% of duodenal ulcers are caused by *Helicobacter pylori (H. pylori)* infection[4]. Long term use of NSAIDs can also cause gastric ulcer. Treatment cost is estimated more than $2 to $4 billion per year. Most of the ulcers heal by using synthetic drugs. After 6-8 wk there is a problem of recurrence of side effects. Therefore, people prefer natural product drugs for disease treatment. Over three quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species at one time or other, have been used for medicinal purposes.

It is estimated that the world market for plant derived drugs may account for about Rs. 200 000 crores. The annual production of medicinal and aromatic plant raw material is worth about Rs. 200 crores. This is likely to touch at US $5 trillion by 2050. WHO reported that 70% of the population of developing countries depends on natural product drugs for health care[5]. *Mentha arvensis* L. (Lamiaceaea) is distributed throughout the western Himalayas and is cultivated throughout world for use as a vegetable. It is an erect aromatic herb that grows up to 60 cm in height with suckers; the stem is cylindrical and the leaves are simple and opposing type. *Mentha arvensis* L. is used as a carminative, anti-spasmodic, anti peptic ulcer agent, and has been given to treat indigestion, skin diseases, coughs and colds in folk medicine. According to several researchers the plant contains 90% mint oil. It contains monoterpenes such as (menthone, menthofuran, methyl acetate cineole and limonene); sesquiterpenes (viridiflorol); flavonoids (luteolin, menthoside, isorhoi folin, rutin hesperidin); phenolic acids (caffeic, chlorogenic and rosmarinic); triterpenes (squalene, a-amyrin, urosolic acid; sitosterol); phytol; tocopherols; carotenoids; choline; betaine; cyclenes; rosmarinic acid; tannin; and minerals[6-8]. Hence the present study is aimed to investigate the antiulcer effect of *Mentha arvensis*.

MATERIALS AND METHODS

Animals

Healthy Swiss Albino rats and mice of the Wister strain weighing 150-200 g and 25-30 g respectively were used for the study. The animals were used with the approval of the Institute animal ethics committee and obtained from Sri Raghavendra enterprises, Bangalore. They were fed with a balanced standard pellet diet and maintained under standard laboratory conditions, providing 24-28℃ temperature relative humidity; 60-70℃, standard light cycle (12 h light, 12 h dark) and water adlibitum (as described by CFTRI, Mysore). Animals were kept in cages with raised floors of wide mesh to prevent coprophagy. Animal welfare guidelines were observed during the maintenance period and experimentation.

Plant material and extraction

The whole herb of *Mentha arvensis* L. were collected from the fields around Gulbarga City, Karnataka in the month of February 2008 and authenticated at the department of Botany, Gulbarga University, Gulbarga. The voucher specimen was kept. The whole plants of *Mentha arvensis* were washed with tap water and shade dried at room temperature in the animal and pharmaceutical Biotechnology laboratory. After 7 d of drying the plant material ground in a pestle and mortar to obtain a fine powder. The powder was weighed and plant powder material was extracted successively using solvents ranging from non-polar to polar i.e. petroleum ether (60-70℃), chloroform (60-70℃) and aqueous (90-100℃), in a soxhlet apparatus for 18 h.

The powdered plant material (120 g) was extracted with 500 mL of petroleum ether. The filtrate gave a light green jelly syrup (3 g) with (w/w) yield of 2.5%. One hundred and twenty grams of plant material was extracted with 500 mL of chloroform. The filtrate gave a green jelly syrup (4.85 g) with (w/w) yield of 4.04%. Similarly, 120 g of powdered material was extracted with 500 mL of water. The filtrate gave a brown jelly syrup (11.5 g) with (w/w) yield of 9.58% en and was administered suspended in 1% Tween 80.

Phytochemical screening

The various extracts of *Mentha arvensis* L. were subjected to qualitative tests for preliminary phytochemical screening. This was carried out by the method described by Harborne and Kokate[9,10] to show the presence of various compounds (flavonoids, alkaloids, tannins, saponins, carbohydrates *etc*) and by Thin Layer Chromatography (TLC). TLC plates were made of silica gel G on glass 20 cm × 20 cm using a solvent system mixture composed of chloroform: methanol:n-propanol:water 5:6:1:4 v/v ratio[11]. The plates were sprayed with a specific reagent and to observe the mobile phase of constituents we used Dragendorff's reagents (alkaloids), polyethylene glycol reagent (flavonoids), 5% ferric chloride solution in methanol with 1% gelatin solution and iodine vapours (tannins), anisaldehyde - sulphuric acid reagent (saponins and triterpenes).

Drugs and chemicals

The chemical used and other solutions were all of analytical grade. All drugs and reagents were prepared immediately before use. The following drugs/chemicals were used: absolute ethanol, hydrochloric acid, solvents, ranitidine. All drugs/chemicals were purchased from Sri. Venkateshwar Chemicals, Gulbarga.

Acute toxicity studies

The method of Lorke[12] was modified to evaluate the oral acute toxicity (LD_{50}) of the various extracts of *Mentha arvensis*. The Swiss albino mice used in the study were starved of food but allowed an excess of water 24 h prior to the study. The oral administration of widely differing doses of the various extracts of *Mentha arvensis* L., (10, 100, 1000, 2000 mg/kg *po*) to four groups of mice (n = 4) was done to establish the range of doses of extracts that would produce toxic effects. This was done by observing the mice over a 72 h period post-treatment for behavioral signs such as excitement, nervousness, dullness, alertness, ataxia or even death. The adopted method estimated LD_{50} by calculating the geometric mean of the dose that caused 100% mortality and the dose which caused no lethality at all.

Induction of acute gastric mucosal damage

Antiulcerogenic activity was evaluated by using three different assay models. Ibuprofen plus pylorus-ligation, administration of 0.6 mol/L HCl and administration of ethanol in different set of rats/mice were employed for induction of acute gastric mucosal lesion.

Antiulcerogenic activity

Ibuprofen plus pyloric ligation ulcer model[13]: In this ulcer model rats weighing 150-200 g were used. They were divided into 5 groups, each group containing six rats. Group 1: This group received 1% Tween 80 5 mL/kg body weight and ibuprofen 200 mg/kg for 5 consecutive days and were considered as a negative control group. Group 2: This group received 375 mg/kg body weight petroleum ether extract and 200 mg/kg body weight ibuprofen for 5 d and were considered as test group 1. Group 3: This group received 375 mg/kg body weight chloroform extract and 200 mg/kg body weight ibuprofen for 5 d and were considered as test group 2. Group 4: This group received 375 mg/kg body weight aqueous extract and 200 mg/kg body weight ibuprofen for 5 d and considered as test group 3. Group 5: This group received the standard drug ranitidine 60 mg/kg body weight and 200 mg/kg body weight ibuprofen for 5 d and were considered as a control group.

Ulcer were induced by the NSAID ibuprofen in all groups of rats 30 min after treatment with 1% Tween 80 (negative control). Different plant extracts suspended in 1% Tween 80 and standard ranitidine were administered orally by a intragastric catheter tube for 5 d. On the 6th day fasted rats were subjected to pylorus ligation under ether anaesthesia. Water was withheld during the postoperative period. The abdomen was opened and the pyloric end of the stomach was ligated and replaced. The abdomen wall was closed by 2-3 interrupted sutures. A 6th dose of 1% Tween 80/extracts/standard drugs was given 30 min prior to pylorus ligation. After 4 h of pylorus ligation the animals were sacrificed and the stomach was isolated after suturing the lower esophageal end. Gastric juice was collected and filtered through glass wool in a measuring cylinder and the stomach was opened along the greater curvature. The gastric contents were centrifuged at 3000 rpm for 5 min, and the supernatant was used for the estimation of total acidity (pH). The volume of gastric juice was expressed as mL/100 g of body weight[14].

For estimation of total acidity[15], 1 mL of supernatant was diluted to 10 mL with distilled water. The solution was titrated against the 0.05 mL/L NaOH using phenolphthalein as an indicator[16]. Titration was continued until the color changed to light pink. The volume of NaOH required was noted and was taken as corresponding to the total acidity. Acidity was expressed as Total acidity = (Volume of NaOH × Normality × 100)/0.1 (mEgq/L). The ulcer score was determined by using a 10 × magnifying hand lens. The scoring of severity of ulceration was as follows[17,18]: 1 mm (pin point) = 1; 1-2 mm = 2; > 2 mm = 3; > 3 mm = 4. The mean ulcer score was determined by dividing the total ulcer indices in a group by the total number of animals in that group[19]. Ulcer Score = Total ulcer index (UI) in a group/Total number of animals in that group. The curative ratio of an ulcer was determined by subtracting the test ulcer score from the control ulcer score divided by the control ulcer score. The result was multiplied by 100[20]. Curative ratio = [(Control ulcer score)-(Test ulcer score)]/(Control ulcer score) × 100.

0.6 mol/L HCl induced ulcer model[21]: Thirty rats were randomly divided into 5 groups; each group contained six rats. Prior to the experiment animals were fasted for 24 h, and fed water ad libitum. Animals in group 1 were treated with 1% Tween 80 (5 mL/kg body weight) and served as untreated control. Animals in Group 2, Group 3, and Group 4 were treated with petroleum ether extract, chloroform extract and aqueous extract (375 mg/kg body weight) respectively. Group 5 animals were treated with the standard drug ranitidine (60 mg/kg body weight). All treatments were performed intragastrically *via* stomach tube. Thirty minutes after pre-treatment with 1% Tween 80, extract, ranitidine treated animals were again intubated using a stomach tube and were given 0.6 mol/L HCl *via* the stomach tube (1 mL/rat). The animals were sacrificed 4 h after the induction of ulcer with HCl in a chloroform chamber. Each animal's abdomen was opened; the stomach was exteriorized and opened through the greater curvature, rinsed under a stream of water, laid out on a flat surface and examined for the presence of mucosal lesions (on ulceration using 10 × magnifying hand lens). Ulcer scores were determined as described in Ibuprofen plus pylorus ligated rats.

Ethanol induced ulcer model[22]: Mice were divided into 5 groups; each group consisted of six animals. Group 1:

Table 1 Effect of *Mentha arvensis* on ibuprofen plus pyloric ligation ulcer model (n = 6, mean ± SE)

Group No.	Treatment	Dosage (mg/kg) B.wt	Gastric contents				% of inhibition
			Vol. of gastric juice (mL/100 g)	pH	Total acidity (mEq)	Ulcer score	
1	Control 1% Tween 80	5 mL	2.08 ± 0.05	2.01 ± 0.04	49.3 ± 0.49	37.1 ± 0.87	-
2	Petroleum ether extract	375	1.93[a] ± 0.06	2.65[b] ± 0.22	40.0[a] ± 0.57	17.3[b] ± 0.42	53.4
3	Chloroform extract	375	1.58[a] ± 0.04	2.93[a] ± 0.03	30.5[b] ± 0.56	15.1[b] ± 0.79	59.2
4	Aqueous extract	375	1.23[d] ± 0.09	3.33[b] ± 0.05	20.3[b] ± 0.33	12.0[d] ± 0.57	67.0
5	Ranitidine	60	0.96[d] ± 0.05	3.78[b] ± 0.13	11.1[b] ± 0.30	11.1[d] ± 0.30	68.7

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* Group 1.

This group received 1% Tween 80 (5 mL/kg body weight) orally. Group 2: this group was treated with petroleum ether extract of plant *M. arvensis* 375 mg/kg body weight. Group 3: this group was treated with chloroform extract 375 mg/kg body weight. Group 4: this group received aqueous extract at a dose of 375 mg/kg body weight. Group 5: animals received standard drug rantidine at a dose of 60 mg/kg body weight. After 30 min of treatment with 1% Tween 80, plant extracts or ranitidine, animals received 1 mL of 90% ethanol *via* an intragastric catheter tube. After 4 h of induction animals were scarified by excess anesthesia. A ventral medline incision was made on each animal and the stomach exteriorized, opened through the greater curvature, rinsed, laid out on a flat surface and examined for the presence of mucosal lesions by 10 × magnifying lens. Ulcer score and curative ratio were determined as described in previous ulcer models.

Statistical analysis

All values were expressed as mean ± SE. Statistical analyses of data were performed using a one-way analysis of variance (ANOVA). A value of $P < 0.005$ was considered statistically significant.

RESULTS

As part of this pharmacological study, the various extracts of *M. arvensis* were first investigated for toxicity in mice. A single oral dose of 2 g/kg of each different plant extract of *M. arvensis* did not produce any signs or symptoms of toxicity in treated animals. Seventy-two hours after administration, no animal died and no significant macroscopic changes occurred. This result probably indicates that the plant extract has no acute toxicity in mice. For this reason, a 5-fold lower dose was used as the maximum dose in all experiments to determine the general antiulcer profile of various extracts of the plant *M. arvensis*.

Effect on ibuprofen plus pyloric ligated ulcer model

The results are depicted in Table 1, which shows a decrease in ulcer score, volume of acid secretion, total acidity and pH in various extracts of *Mentha arvensis* i.e. aqueous extract, petroleum ether extract and chloroform extract. There was also a significant reduction in these parameters in the ranitidine treated ulcer group when compared to the negative control group.

In the group of animals in which ulcers were induced using ibuprofen and pylorus ligation, the aqueous extract showed significant activity in all the selected parameters with 67% inhibition of ulcers and a significant reduction in total acidity, ulcer score and gastric secretion ($P < 0.001$). Standard drug treatment with ranitidine (60 mg/kg) also showed significant reductions in acidity, gastric secretion and ulcer score with a curative ratio of 68.7% when compared to negative control group. The petroleum ether extract and chloroform extract produced curative ratios of 53.4% and 59.2%, respectively (Table 1).

Effect on 0.6 mol/L HCl induced ulcer model

The ulcer indices in rats treated with ranitidine and aqueous extract of *Mentha arvensis* were found to be significantly ($P < 0.001$) lower than that of control treated rats with a curative ratio of 71.2% and 67.5%, respectively. In addition rats that received the chloroform and petroleum ether extracts showed reductions in ulcer indices with a curative ratio of 57.4% and 50.5% respectively compared with 1% Tween 80 treated control rats (Table 2).

Effect on ethanol induced ulcer model

The aqueous extract of *Mentha arvensis* and ranitidine significantly increased the macroscopic curative ratio to 65.4% and 69.75%, respectively, compared to control groups. The ulcer index was significantly reduced in mice pretreated with chloroform and petroleum ether extract, with a curative ratio 62.9% and 53.11%, respectively, compared to the negative control group (Table 3).

DISCUSSION

Peptic ulcer disease is a chronic inflammatory disease characterized by ulceration in the upper gastro-intestinal tract[23]. The pathophysiology of ulcers is due to an imbalance between aggressive factors (acid, pepsin, *H. pylori* and NSAIDs) and local mucosal defensive factors (mucus bicarbonate, blood flow and prostaglandins). The integrity of the gastroduodenal mucosa is maintained through a hemostatic balance between these aggressive and defensive factors. The major cause of gastric ulcer is the chronic use of NSAIDs. Therapeutic and adverse effects of NSAIDs have been attributed to the ability of these drugs to inhibit the action of cyclooxygenase (COX). COX is responsible for the synthesis of prostaglandins

Table 2 Effect of *Mentha arvensis* on 0.6 mol/L HCl induced ulcer model (n = 6, mean ± SE)

Group No.	Treatment	Dosage (mg/kg) B. wt	Ulcer score	% of inhibition
1	Control 1% Tween 80	5 mL	31.3 ± 0.42	-
2	Petroleum ether extract	375	15.5[a] ± 0.50	50.5
3	Chloroform extract	375	13.3[a] ± 0.42	57.4
4	Aqueous extract	375	10.1[b] ± 0.47	67.5
5	Ranitidine	60	9.0[b] ± 0.44	71.2

[a]P < 0.05, [b]P < 0.001 *vs* Group 1.

Table 3 Effect of *Mentha arvensis* on 90% Ethanol induced ulcer model (n = 6, mean ± SE)

Group No.	Treatment	Dosage (mg/kg) B.wt	Ulcer score	% of inhibition
1	Control 1% Tween 80	5 mL	27.0 ± 0.36	-
2	Petroleum ether extract	375	12.6[a] ± 0.95	53.1
3	Chloroform extract	375	10.0[b] ± 0.68	62.9
4	Aqueous extract	375	9.33[d] ± 0.76	65.4
5	Ranitidine	60	8.16[d] ± 0.47	69.7

[a]P < 0.05, [b]P < 0.01, [d]P < 0.001 *vs* Group 1.

that normally inhibit acid secretion, as well as having a protective effect on the gastric mucosa. Infection of the stomach mucosa with *H. pylori* - a gram-negative spiral shaped bacterium - is now generally considered to be a major cause of gastrointestinal ulcers. Treatment includes H2-receptor antagonists (cimetidine), proton pump inhibitors (omeprazole) and cytoprotectives (misoprostol). Antacids, like aluminum hydroxide and magnesium hydroxide, are used often to neutralize excess gastric acidity in the stomach. Due to problems associated with recurrence after treatment, there is the need to seek an alternative drug against gastrointestinal ulcers[24].

The present investigation demonstrated the efficacy of *Mentha arvensis* plant extract against gastric ulceration induced by 3 experimental models viz., ibuprofen induced gastric ulceration, 0.6 mol/L HCl induced ulceration and 90% ethanol induced ulceration. The plant extract *Mentha arvensis* and standard drugs produces a decrease in the ulcer number, total acidity, volume of gastric juice and pH in the ibuprofen induced pyloric ligation ulcer model in rats. The curative ratio in this pyloric ligation model was 53.4%, 59.2%, 67% and 68.7% using petroleum ether, chloroform, aqueous extract and standard drug ranitidine, respectively. This indicates that the plant has antiulcerogenic, antisecretory and cytoprotective actions. Several investigators have reported the same results after plant extract treatment. Gastric mucus is known to protect the gastric mucosa against tissue damage by HCl produced by parietal cells. It consists of viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that covers the entire gastrointestinal mucosa. Moreover, mucus is capable of acting as an antioxidant thus can reduce mucosal damage mediated by oxygen free radicals. The protective properties of the mucus barrier depend not only on gel structures but also on the thickness of the layer covering the mucosal layer[25]. A decrease in gastric mucus renders the mucosa susceptible to injuries induced mainly by acids, NSAIDs and alcohol.

The effect of Mentha aervensis extracts on the mucosal damage in the 0.6 mol/L HCl induced gastric ulcer model in rats reveals the decreases in ulcer scores. Treatment with successive extracts and standard drug shows the decreases i.e. petroleum ether (50.5%), chloroform (57.4%), aqueous (67.5 %) and ranitidine (71.2%). This indicates that the extracts have cytoprotective effects against the irritant actions caused by acids. Studies of plant extract *Tephrosia* purpurea treated ulcerogenic rats have also shown cytoprotective activity[26].

Peptic ulcer is an imbalance between gastroduodenal mucosal defense mechanisms and offensive factors. Some studies have revealed that reactive oxygen species (ROS) and lipid peroxidation are implicated in the pathogenesis of ethanol induced gastric lesions and gastrointestinal damage and that they attack and damage many biological molecules such as prostaglandins. After an initial reaction with ROS, a continuing chain reaction causes cell injury and ultimately cell death[27-31]. Therefore, treatment with antioxidants and free radical scavengers can decrease ethanol induced gastric mucosal damage. In the present study, a reduction in ulcer number in ethanol induced gastric ulceration in mice was found after various extract treatments, such as petroleum ether (53.1%), chloroform (62.9%), aqueous extract (65.4%) of *Mentha arvensis* and the standard drug ranitidine (69.7%). This indicates cytoprotective actions in the plant extracts. Plant chemical substances such as flavonoids, tannins, terpenoids *etc* have been shown to scavenger free radicals and therefore are viewed as promising therapeutic drugs for free radical pathologies. Phytochemical tests revealed the presence of flavonoids and terpenoids in the extracts of *Mentha arvensis*. Some of the triterpenes are known as an antiulcer agents and their action has been mentioned to be due to activation of cellular proteins, reduction of mucosal prostaglandin metabolism, cytoprotective actions and reduction of gastric vascular permeability[32-34]. However, the mechanism by which this extract produces an antiulcer effect is not entirely clear. The results in present study seems to provide support for the use of *Mentha arvensis* as an antiulcer drug in folk medicine. Therefore, also in view of its large use in India more detailed phytochemical and pharmacological investigations on the antiulcer effects and toxicity studies are required. In all three ulcer experimental models the aqueous extract shows the best antiulcerogenic action, due to the presence of tannins and flavonoids, as in literature references.

The present data obtained from various extracts

of *Mentha arvensis* L. showed the presence of a gastro-protective effect and improved ulcer healing properties. The data also confirmed the traditional claim on the use of *M. arvensis* for treating gastric ulcers in the Indian subcontinent. Although at this time it is difficult to explain the exact mechanism involved with these crude extracts, the effects obtained on acute and chronic gastric lesions suggest a multifactorial mechanism, involving *M. arvensis* influence on free-radical scavenging properties, on endogenous prostaglandins and sulphydryl groups.

COMMENTS

Background

The stomach defends itself from hydrochloric acid and pepsin by creating a mucus coating (that shields stomach tissue), by producing bicarbonate and by circulating blood to the stomach lining to aid in cell renewal and repair. When these functions are impaired it can lead to the formation of an ulcer. Mentha arvensis has plenty of medicinal property which contains corn mint used for the cure of stomach cancer and peptic ulcer.

Research frontiers

Mentha arvensis is a carminative and antispasmodic herb has excellent good results in the treatment of indigestion, biliousness, flatulent colic, *etc*. the present study is used for the prevention of the peptic ulcer with mentha arvensis, the present research shows the efficiency of the menthe arvensis for the treatment of the peptic ulcer.

Innovations and breakthroughs

Earlier the mentha arvensis has a carminative, cholatotic, secretolytic and cooling properties. Mint oil has antimicrobial activity and contain corn mint which is effectively used for the treatment of stomach cancer. The present investigation is aimed to study the antiulcer activity of mentha arvensis. In this research three solvent's extract were used such as petroleum ether, chloroform and aqueous. Among all the three extract, aqueous extract has shown/given good result for the cure of ulcer when it compared with the standard drug available in the market.

Applications

Pyloric ligation ulcer model in rats. The results of the present article suggest that the plant mentha arvensis has a bioactive compounds which can dissolve in solvents like petroleum ether, chloroform and aqueous which can be used in the preparation of drugs for the treatment of intestinal ulcer.

Terminology

The intestine has mucus coating, a sort of defensive /protective coverage against intestinal enzymes, Mentha arvensis has flavanoids, alkaloids, and terpenoids which will acts as gastro-protective agent's. These compounds are more potent than the standard drug available in the market.

Peer review

The present investigation on bioactive compounds of mentha arvensis plant in which authors have analyzed the preventive property of peptic ulcer by using secondary metabolites of the plant. The results reveals that there is significant efficacy of mentha arvensis plant extract against gastric ulceration induced by three experimental model viz Ibuprofen induced gastric ulceration, 0.6 mol/L HCl and 90% ethanol induced ulceration. Various extracts of mentha arvensis plant and standard drug both have shown decrease in the ulceration, total acidity, volume of gastric juice and pH in Ibuprofen induced pyloric ligation ulcer model in rat. Therefore, This study helps to use phyto compounds as novel. Drug for the treatment of gastric ulcer.

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CASE REPORT

Ileal inflammatory fibroid polyp causing chronic ileocolic intussusception and mimicking cecal carcinoma

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Abstract

Inflammatory fibroid polyp (IFP) is a rare, idiopathic pseudotumorous lesion of the gastrointestinal tract. While mostly reported as solitary gastric lesions, multiple cases of small bowel IFPs are also reported. It is a documented cause of intussusception in adults. In the case reports of ileal inflammatory fibroid polyps with intussusception, an emergent presentation with small bowel obstruction has been most often described. Here we depict a case of ileal inflammatory fibroid polyp presenting with chronic intermittent ileocolic intussusception, anemia and weight loss with an endoscopic appearance mimicking necrotic cecal carcinoma.

Key words: Ileal polyp; Colon cancer; Endoscopy; Intussusception; Intussusception in elderly

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INTRODUCTION

Inflammatory fibroid polyp (IFP) is a rare, idiopathic pseudotumorous lesion of the gastrointestinal tract, first described by Vanek in 1949 as an eosinophilic submucosal granuloma[1]. In that first report of six gastric lesions, Vanek called attention to the inflammatory nature of the lesions and their submucosal origin. In 1953 Helwig and Ranier confirmed the fibroblastic origin of proliferating spindle and stellate cells, and coined the term inflammatory fibroid polyp which has remained the generally accepted term[2]. Subsequently, these lesions were found throughout the gastrointestinal tract[3].

Here we describe a case of ileal inflammatory fibroid polyp presenting with chronic intermittent ileocolic intussusception, anemia and weight loss with an endoscopic appearance mimicking necrotic cecal carcinoma.

CASE REPORT

A 76 year old diabetic female with chronic GERD was referred in September 2007 for screening colonoscopy. Since cholecystectomy a year earlier, she noted loose bowel motions. Rarely, she noted blood on the toilet tissue but denied any abdominal pain or weight loss. The patient was slightly overweight. Abdominal examination did not reveal any masses or tenderness. CBC at that time revealed a normal hemoglobin level.

The patient underwent screening colonoscopy, which was normal (Figure 1A) except for external hemorrhoids, scattered diverticulae in the right colon and two diminutive, non-neoplastic polyps in the sigmoid colon.

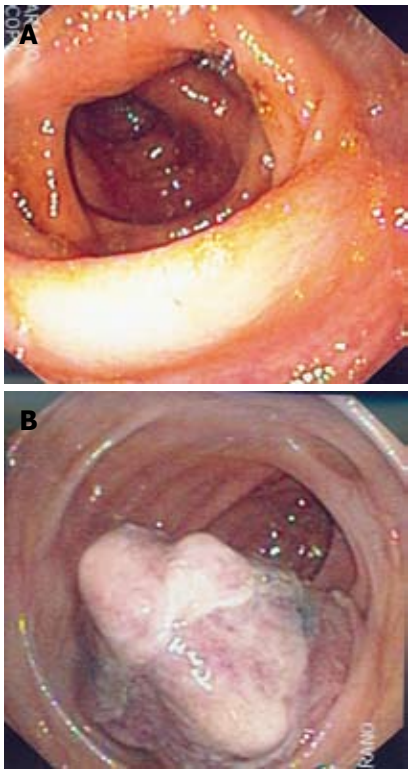

Figure 1 Patient underwent screening colonoscopy. A: Initial screening colonoscopy with no visible mass at the level of the ileocecal valve; B: Repeat colonoscopy revealing the polypoid cecal mass.

Esophagogastroduodenoscopy revealed a small hiatus hernia and non-specific gastritis. Pathology revealed that the colonic polyps were hyperplastic. Stomach specimens showed H pylori negative moderate chronic gastritis with no metaplasia. She was advised to continue omeprazole over the counter daily for symptomatic relief, and cellulose was added for presumed bile acid diarrhea.

Four months later, in January 2008, she first experienced epigastric pain which by the time of her clinic visit was described as "upset stomach". Her abdominal examination was unremarkable and she reported improvement in diarrhea with cellulose. At this time, esomeprazole was substituted for omeprazole.

In May 2008, eight months after her initial endoscopy, she again had an episode of abdominal pain but this time it was associated with unheralded hematochezia. Both were self-limited and when seen in the clinic a few days later, she was noted to be pale but vital signs were within normal limits and abdominal examination revealed no tenderness or masses. Hemoglobin was now 6.7 gm/dL. She was transfused with 2 units of packed red blood cells as an outpatient, and oral iron therapy was begun.

At the next follow-up visit in a few weeks, the patient again complained of abdominal bloating and diarrhea. Hemorrhoidal-type rectal bleeding was again noted. Her hemoglobin was 10.1 gm/dL. Her weight now was 119 lb, representing an 11 lbs weight loss since her presentation. Abdominal exam at this time revealed a movable fullness in the right lower quadrant, which was

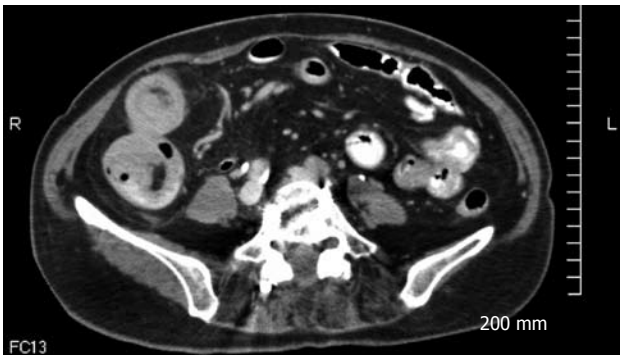

Figure 2 Pre-operative computerized tomography scan showing the right colonic intussusception.

presumed to be fecal content. Follow-up examination two weeks later failed to confirm the abdominal mass; however her hemoglobin was 9.7 gm/dL. Because of her continuing anemia, vague abdominal symptoms and weight loss, an overlooked colon lesion was considered and repeat colonoscopy recommended.

At endoscopy two weeks later, a 6-7 cm exophytic, polypoid cecal mass (Figure 1B) was seen. Biopsy of this mass was read as "inflammatory exudate with no mucosal tissue fragments or malignant cells identified".

The patient was then referred to General Surgery with a presumptive diagnosis of overlooked cecal carcinoma. Preoperative clinical evaluation revealed no palpable mass, normal labs and carcinoembryonic antigen level except for anemia with hemoglobin of 9.5 gm/dL. Preoperative abdominal/pelvic CT scan with oral and intravenous contrast revealed a "right colonic intussusception with a low density 2.2 × 2.7 cm mass as the lead point consistent with the patient's known colon carcinoma" (Figure 2). An excision was then planned with open laparotomy to establish a definitive diagnosis. Intraoperatively, a small bowel intussusception was seen with a polypoid ileal tumor being the lead point. No other lesions were seen. The location of this tumor coincided with the one seen on colonoscopy.

The excised bowel segment revealed an ulcerated polypoid mucosal mass (Figure 3) in the ileum, 25 cm from the distal colonic margin and 7.7 cm from the proximal ileal margin. It measured 5.5 cm × 3.5 cm × 2.5 cm in size. There was no muscle wall invasion and the uninvolved mucosa was unremarkable except for an incidental colonic tubular adenoma. Microscopically, the tumor was well-vascularized and composed of sparsely cellular proliferation of bland spindled cells with fibromyxoid background containing numerous eosinophils (Figure 4). The spindle cells were negatively stained for CD117 (c-kit), smooth muscle actin, and CD34. The final pathologic, microscopic diagnosis was "inflammatory fibroid polyp".

The patient did well post-operatively, remaining free of symptoms following discharge. Her hemoglobin gradually normalized with oral iron in the weeks following her surgery.

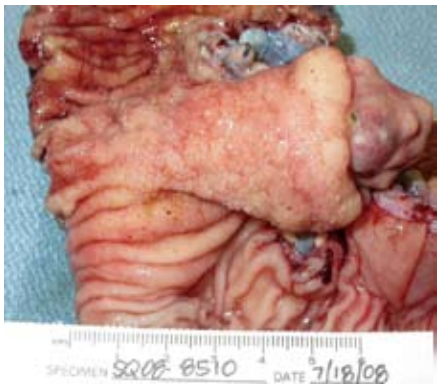

Figure 3 Polypoid mucosal mass in the excised bowel segment showing surface ulceration.

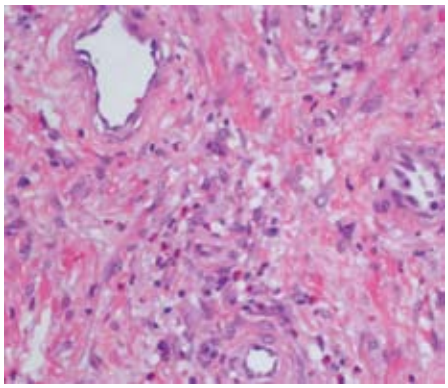

Figure 4 The tumor is composed of bland spindle cells with fibromyxoid background containing numerous eosinophils (× 400).

DISCUSSION

Inflammatory fibroid polyp is a rarely seen non-tumorous lesion of the gastrointestinal tract. Histologically it is characterized by an admixture of numerous blood vessels, fibroblasts and edematous connective tissue accompanying a marked cellular infiltrate which contains eosinophils[1]. The histologic differential diagnosis includes gastrointestinal stromal tumor (GIST) and inflammatory myofibroblastic tumor, which stains for CD117 and smooth muscle actin, respectively. CD34 staining is present in variable numbers of inflammatory fibroid polyps and inflammatory myofibroblastic tumors[4,5]. All IFPs lacked c-kit staining and no mutations were identified, thereby helping to exclude malignancy[6].

Grossly, lesions may be polypoidal or sessile, varying in size from 0.2-12 cm with an average reported size of 4 cm[5-7]. They usually present in the sixth or seventh decades but cases were reported in a wide age range of 2 to 90 years[5-7]. The true prevalence of IFPs is not known. While mostly reported as solitary gastric lesions, multiple cases of small bowel lesions were subsequently described[1,7-10]. In Johnstone and Morson's series of 76 cases, 18% were in the small bowel[3]; a Spanish study of 26 cases by Acero *et al*[11] revealed 7 ileal and 2 jejunal IFPs. In a literature review, Bays *et al*[12] cited fewer than 20 jejunal IFPs had been reported until 2004. In general, the small bowel has been reported to be the second most common site of IFP occurrence after the stomach; however, Ozolek *et al*'s series of 42 cases revealed 13 cases were in the large bowel and 10 in the small bowel[6].

In the case reports of ileal inflammatory fibroid polyps with intussusception, an emergent presentation with small bowel obstruction has been most often described[7,10,13-19]. One patient was described with chronic diarrhea and another with anemia as the only sign[20,21]. While adult intussusception represents only 5% of all cases of intussusception, unlike children, 90% of adults with intussusception have an underlying lesion, nearly half of which are malignant[22,23]. Benign causes of intussusception include Meckel diverticulum, lipomas, neurofibromas, endometriosis and appendiceal stump in a patient who has undergone appendectomy[24,25].

Our patient's presentation was unusual because of the absence of pain even during intussusception as noted both at colonoscopy and pre-op CT scanning. In retrospect, the intermittent right lower quadrant mass was almost certainly the palpable intussusceptum. The necrotic tumor at colonoscopy was most likely the lead point of the IFP which was engorged or ischemic. We believe that the likely cause for her hematochezia was the IFP since the anemia, unresponsive to iron therapy preoperatively, normalized postoperatively.

Our case illustrates that in some patients, symptoms may be subtle and nonspecific such that a correct preoperative diagnosis is difficult if intussusception is not considered, as is the case usually. The sensitivity of contrast studies such as barium enema and small bowel series are relatively poor and abdominal ultrasound lacks specificity[26]. However, del-Pozo *et al*[27] reported a crescent-in-doughnut sign that reportedly helps diagnose an intussusception *via* ultrasound. In accordance with other studies, abdominal CT scanning is the most useful study because it reveals a mass with a central dense area and a halo of low attenuation representing the intussusceptum and the edematous intussuscepiens, respectively[23,28-29]. Newer techniques which have recently assisted in the diagnosis include capsule endoscopy and retrograde double balloon enteroscopy[30,31]. Given the risks of underlying malignancy and vascular compromise, once adult intussusception is diagnosed, the treatment is operative resection.

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CASE REPORT

Gastric amyloidoma in patient after remission of Non-Hodgkin's Lymphoma

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Abstract

Amyloidosis is commonly systemic, occasionally organ-limited, and rarely a solitary localized mass. The latter, commonly referred to as tumoral amyloidosis, is described as occurring in nearly every organ/tissue. Only a few reports of gastric amyloidosis exist today. We describe a 72 year-old black male from Barbados presenting with 3 d of diffuse abdominal pain. His medical history included Non-Hodgkin's Lymphoma diagnosed five years ago, status-post six rounds of cyclophosphamide, adriamycin, vincristine, prednisone chemotherapy, and currently was in remission. On computed tomography scan of the abdomen, thickening and calcification of the gastric wall was noted along with pneumatosis. On esophagogastroduodenoscopy, a large circumferential friable mass was seen from the gastroesophageal junction to the body. A large non-bleeding 3 cm polyp was also seen in post bulbar area of duodenum. Biopsies were stained with Congo red and gave green birefringence under polarized light, consistent with tumoral amyloidosis. Positron emission tomography scan revealed diffuse gastric mucosa uptake compatible with gastric malignancy without metastatic foci. Treatment for gastric amyloidomas has presently been one of observation or, at most, resection of the amyloid mass. It is not known if our patient required the same approach or if this warranted the re-institution of chemotherapy for Non-Hodgkin's Lymphoma. Until more reports of tumoral amyloidosis are made known, treatment as well as prognosis remain uncertain.

Key words: Amyloidoma; Non-Hodgkin; Lymphoma; Stomach; Duodenum

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INTRODUCTION

In 1842, Rokitansky described a disease phenomenon known today as amyloidosis[1]. Amyloidosis has been classified as primary or secondary, being predisposed by infection, inflammation, or systemic disease. It is a process involving the deposition of proteinaceous fibrillar material in a pattern that is typically systemic, occasionally organ-limited, and very rarely as solitary localized mass. The latter, commonly referred to as tumoral amyloidosis, can often masquerade as a neoplasm and has been reported to have occurred in nearly every organ or tissue including the brain, stomach, esophagus, jejunum, heart, cervical spine, thoracic spine, urethra, bladder, popliteal fossa, as a nodule on a lower extremity, breast, parotid gland, and submandibular gland[2-17]. Gottfried *et al*[18] reported amyloidoma of the trigeminal nerve mimicking as a tumor of the cavernous sinus.

Amyloidoma of the stomach was described in 1956 by Intriere and Brown[19]. In 1978 the first autopsy-proven case of tumoral amyloidosis was reported[20]. Only a few

more cases have been published since then. In the case below, we describe tumoral amyloidoma confined to the stomach and duodenum in a male who had been in remission for Non-Hodgkin's Lymphoma (NHL).

CASE REPORT

A 72 year-old black male with a history of NHL sought medical attention after a three day course of diffuse abdominal pain. The pain was exacerbated by seating upright. The patient also complained of early satiety and a 60-lb weight loss over the last year prior to presentation. He denied dysphagia, odynophagia, or change in bowel habits or character of stool. The patient had a colonoscopy as well as an upper endoscopy six years ago at an outside institution which he stated was unremarkable.

The patient was treated for his NHL five years ago, with six rounds of status-post six rounds of cyclophosphamide, adriamycin, vincristine, prednisone chemotherapy, and currently was in remission chemotherapy, and was believed to be in remission upon presentation. There was no family history of gastrointestinal disorders or malignancy. His only medication was pregabalin which was prescribed to him for lower extremity pain and weakness. The patient had immigrated from Barbados and had been living in United States for the past 40 years. He had been a life-long smoker and consumed a moderate amount of alcohol daily.

His physical exam was pertinent for mild pallor and generalized abdominal tenderness, but no abdominal masses or adenopathy was appreciated. On laboratory evaluation, a urinalysis and culture was significant for Beta Hemolytic Strep Group B urinary tract infection, but negative for Bence Jones protein. Complete blood count and iron panel revealed anemia of chronic disease. Electrolyte and hepatic panels were unremarkable.

On admission, a computed tomography scan of the abdomen revealed thickening and calcification of the gastric wall with associated pneumatosis, as well as a 1 cm pedunculated mass in the proximal duodenum (Figure 1). No focal hepatic masses or biliary ductal dilatations were seen. He subsequently underwent an esophagogastroduodenoscopy (EGD), whereby a large circumferential friable, ulcerated, nodular mass was seen from the gastroesophageal junction to the body of the stomach. A large non bleeding ulcer was appreciated in the fundus of the greater curvature. Lastly, a non-bleeding 3 cm polyp was noted in post bulbar area of the duodenum (Figure 2). Biopsies were obtained and, due to their appearance under light microscopy with Hematoxylin and Eosin stain, were stained with Congo red (Figure 3). This showed a classic green birefringence under polarized light, consistent with tumoral amyloidosis. A positron emission tomography scan revealed diffuse gastric mucosa uptake compatible with gastric malignancy but no hypermetabolic neoplastic foci elsewhere (Figure 4).

After medically controlling his abdominal pain, the patient was discharged home with a plan to observe his condition periodically.

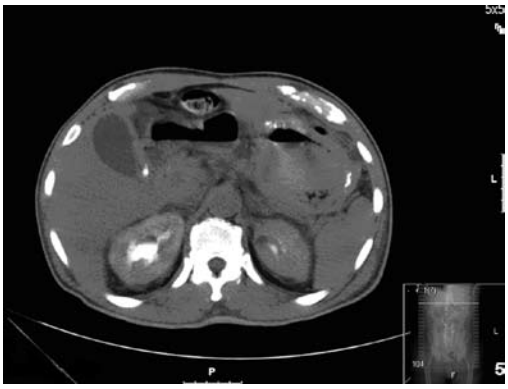

Figure 1 CAT scan of the abdomen revealed thickening and calcification of the gastric wall with associated pneumatosis, as well as a 1 cm pedunculated mass in the proximal duodenum.

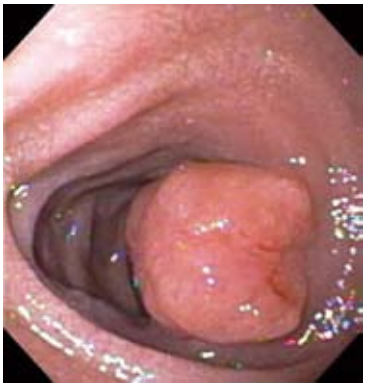

Figure 2 On esophagogastroduodenoscopy, a non bleeding 3 cm polyp was noted in the post-bulbar area of the duodenum.

DISCUSSION

Multiple diagnostic modalities have been employed in verifying amyloidosis. The lesion, when subjected to hematoxylin-eosin stain, will appear as diffuse deposits of acellular, amorphous eosinophilic material on light microscopy. When stained with Congo red, green birefringence is observed under polarized light. Amyloid is also described as having a beta pleated sheet arrangement on radiographic diffraction.

Balázs[21] in 1981 performed the first electron microscopy study on amyloidoma biopsies from the stomach corpus. They found three dominant cells: plasma cells, fibroblasts, and myofibroblasts. Mucus secretion was impaired. The cytoplasm of mucin producing cells was filled with a filamentous substance which replaced the organelles. Myofibroblasts located within the amyloid mass were observed to show active secretion of microfilaments.

With regards to pathogenesis, the formation of amyloid was posited to result from an inadequate immune response to an antigenic stimulus resulting in decreasing mucus production in gastric mucosa while increasing the population of myofibroblasts[21]. Hamidi Asl *et al*[22] observed monoclonal immunoglobulin light chains as the principal composition of amyloidomas in the respiratory and urinary tract, thus implicating local monoclonal plas-

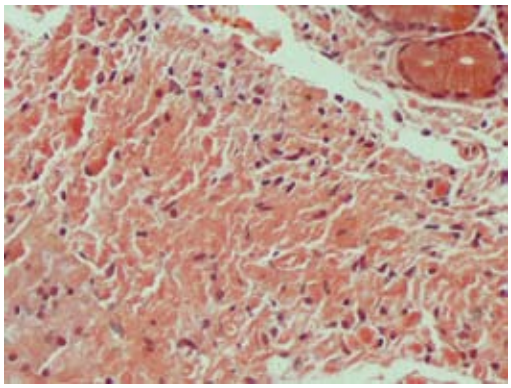

Figure 3 Under light microscopy, gastric biopsy appears salmon-colored when stained with Congo Red.

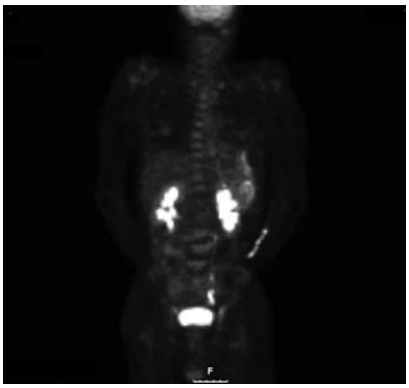

Figure 4 PET scan revealed diffuse gastric mucosa uptake compatible with gastric malignancy but no hypermetabolic neoplastic foci elsewhere.

ma cell proliferation as the underlying cause of the disease process. Regarding amyloidoma of the small bowel, Saindane *et al*[23] theorized that amyloid deposition into mesenteric vasculature induces a focal ischemia leading to wall thickening, edema, hemorrhage, and ulceration thus predisposing to significant amyloid deposition.

There is a paucity of cases in the literature of localized amyloid depositions in the stomach to date, and the physical descriptions of such cases varies. Amyloidosis of the stomach has been described as flat circular lesions with a fine granular appearance[24], diffuse thickening of the gastric wall with a tendency to bleed[20,25], irregularly-shaped, soft, mural tumors[21], and even as a gastric ulcer with heaped up, friable edges, masquerading as malignancy[26]. In addition, loss of rugal folds and antral narrowing have been reported[27,28]. Our patient's amyloidoma appear as a large circumferential friable mass that was ulcerated, nodular, edematous, and bled easily on contact.

Being such a rare phenomenon, consensus on treatment and the prognosis for amyloidoma, particularly in the gut, is quite limited. In 1978, Ikeda *et al*[20] reported a 68 year-old female with epigastric distress who was worked up for what was believed to be a gastric tumor. The patient was eventually treated with a partial gastrectomy and Billroth I anastomosis only to be finally diagnosed with a localized amyloidosis of the stomach. The patient died ten months later of unrelated causes and, at autopsy, no amyloid deposits were found in the remnant stomach or anywhere else in her body. Another patient underwent subtotal gastrectomy and clearance of perigastric lymph nodes as this was believed to be essential in preventing postoperative recurrence of amyloid deposition[26]. Solanke *et al*[29] described a 48-year-old woman with progressive dysphagia and weight loss who was found to have an amyloidoma of the distal esophagus. She was treated with an esophageal bypass using a segment of colon. Suri *et al*[10] suggested that localized amyloidosis had a good prognosis as compared to systemic amyloidosis with treatment simply by complete resection of the lesion itself. A favorable prognosis was given also to amyloidoma of the pulmonary system[30,31].

Kahi *et al*[4] offered a more conservative approach of careful long-term observation alone as the management of amyloidoma of the esophagus. In one case where gastric amyloidoma presented with dyspepsia, the treatment consisted solely of a proton pump inhibitor and a prokinetic medication[24]. No further treatment was offered and the patient was asymptomatic at a 10-mo follow-up.

In regards to our patient, it is unknown whether the amyloidoma in the gut was primary or secondary to his prior history of NHL. At the time of presentation, he was in remission from NHL after receiving standard chemotherapy treatment and an EGD one year prior to diagnosis of NHL excluded any visible pathology of the stomach. Nonetheless, Krishnan *et al*[32] found half of their subjects with soft tissue amyloidomas had a lymphoproliferative disorder. Therefore, does our observation represent relapse of his prior disease process? No studies exist to answer this question. What implication, if any, does this have for future patients who are being surveyed for NHL relapse? Since most of the literature supports observation or at most, resection of the amyloid mass, does our patient require the same or does this case warrant future treatment? Should the re-institution of chemotherapy for NHL be considered? Only the contribution of similar cases to the literature will elucidate the matter.

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Events Calendar 2009

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19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Acknowledgments

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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

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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

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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]

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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]

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Personal author(s)

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

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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

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

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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

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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

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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/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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