Original Article

Archives of Clinical & Experimental Surgery

Apoptotic Versus Angiogenic Factors in Gastric and Colorectal Cancers

Enas A. Hamed[1], Madeha M. Zakhary[2], Doaa W. Maximous[3], Nagwa S. Ahmed[4], Amany O. Mohamed[2]

Abstract

Objective: Gastric-colon cancer is a complex, multi-stage disease involving deregulation of different signaling cascades. This study was conducted to determine the extent of apoptosis, angiogenesis, inflammation, and oxidative stress in patients with gastric-colon cancers. Plasma levels of soluble (s) Fas, bcl-2 as antiapoptotic indices; cathepsin D (CD), calpain I and II as proteolytic, apoptotic indices; nitric oxide (NO), lipid peroxides (LPER) as oxidative stress, angiogenic indices, and tumor necrosis factor (TNF)-α as apoptotic, inflammatory, angiogenic indices were measured in gastric-colon cancer patients.

Methods: Thirty gastric-colon cancer patients [colorectal (n=20), gastric (n=10) cancers], 30 with benign gastrointestinal tract (GIT) masses and 30 healthy controls, were recruited. sFas, bcl-2 and TNF-α were measured by immunosorbent assay kits, and CD, calpain I and II, LPER and NO by chemical methods.

Results: sFas, bcl-2, CD, calpain I, calpain II, NO, and TNF-α were higher in malignant and benign GIT masses than controls, and in malignant than benign masses. In gastric tumors, calpain I, calpain II, CD, LPER, and NO levels were higher than colorectal. In benign and malignant GIT masses, positive correlations were found between sFas, bcl-2, CD, calpain I, calpain II, LPER, NO and TNF- α.

Conclusions: Gastric-colon malignancy patients exhibited decreased apoptosis, as evident by an increase in antiapoptotic indices, i.e. sFas and bcl-2, and increased angiogenic activity, as evident by enhanced proteolytic activity of cathepsin-D and calpain I and II. These parameters were higher in gastric than colorectal cancers reflecting aggressive behavior of the earlier. Thus, decreased apoptosis and enhanced angiogenesis give growth priority in gastric-colon cancers, and the angiogenic factors' blockage may delay the tumor's spread.

Key words: *Angiogenesis, apoptosis, gastric-colon cancer, inflammation, oxidative stress*

[1]*Departments of Physiology*
[2]*Departments Biochemistry*
Faculty of Medicine
Assiut University
Assiut, Egypt

[3]*Departments of*
Surgical Oncology
South Egypt
Cancer Institute
Assiut University
Assiut, Egypt

[4]*Departments Biochemistry*
Faculty of Medicine
Sohag University
Sohag, Egypt

Received: January 28, 2012
Accepted: February 16, 2012
Arch Clin Exp Surg 2012; 1: 71-84
DOI: 10.5455/aces.20120216121853

Corresponding Author
Enas A Hamed, MD
Department of Physiology
Assiut University
P.O. Box: 71526
Assiut, Egypt
eah3a2010@yahoo.com

INTRODUCTION

Gastric cancer (GC) remains the second most frequent cause of cancer-related death worldwide, accounting for over 10% of global cancer deaths [1]. Colorectal cancer (CRC) is the third most common cancer in the world [2]. Gastric and colorectal cancers are characterized by rapid progression and late clinical presentation. With more frequent use of sensitive imaging techniques in the diagnosis of gastrointestinal (GIT) cancer, people are still intensively interested in identifying body fluid biomarkers that could potentially be useful for prognosticating, and they are conceivable in surveillance [3].

Apoptosis is a fundamental mechanism that regulates cell and tissue homeostasis and eliminates cancer cells. Extrinsic and intrinsic-mediated pathways lead to apoptosis. An extrinsic (receptor) mediated pathway is characterized by activation of caspases following plasma membrane death receptors' stimulation, including those of the tumor necrosis factor (TNF) receptors' superfamily, such as TNFR, Fas, and TRAIL receptors [4]. In an intrinsic mediated pathway, mitochondria will be disrupted by cell stress, releasing cytochrome C into cytoplasm. This leads to converting procaspase-9 into caspase-9, which activates caspase-3, and triggers an irreversible apoptotic program. This family includes proapoptotic members as Bax, Bid, Bad, Bim, and Bmf, and antiapoptotic members as bcl-2 and bcl-xL [5]. Fas (Apo-1/CD95) is a cell surface protein receptor that induces apoptosis through its cytosolic tail after binding to Fas ligand (FasL). The Fas-FasL system plays an important role in cytotoxicity against cancer cells. Both Fas and FasL exist as membrane-bound and soluble forms. Soluble Fas (sFas) binds to and neutralizes FasL, thereby antagonizing the Fas-FasL pathway, leading to malignancy development and progression [4]. Previous studies conducted on sFas in sera of GC patients are rather contradictory. While Yatsuya et al. [6] reported increased sFas serum levels only in female GC patients than controls, Liang et al. [7] reported such increased levels in all patients. Studies conducted on bcl-2 serum levels in GIT tumor patients are few and mainly dependent on immunohistochemistry [8]. Giannoulis et al. [9], using ELISA, reported significant increased bcl-2 serum levels in patients with CRC. Targeting sFas and bcl-2 in tumor cells can enhance other routine therapeutic technique effects.

Apoptotic pathways independent of caspases also exist. Cathepsin D (CD) refers to lysosomal proteases belonging to aspartic endopeptidases that mediate apoptotic cell death induced by TNF-α and cytotoxic drugs. Cathepsin D plays roles in intracellular protein catabolism, extracellular matrix (ECM) components degradation, and activation of some proteases. Up-regulation of CD in certain tumor tissues induces cancer cells escaping from the primary site, and breaks down ECM connective barriers and the basement membrane [10]. An increased CD serum level was reported in large intestine malignancy patients [11]. Its role in CRC apoptosis and angiogenesis remains to be determined.

Calpains are a family of calcium-activated non-lysosomal neutral cysteine endopeptidases, which limit proteolysis of substrate proteins. Calpain-I (μ-calpain) and calpain-II (m-calpain) are two major typical calpain isoforms. Calpastatin functions as a specific endogenous inhibitor for calpain-I and calpain-II. Numerous lines of evidence demonstrated that calpains were involved in oncotic cell death [12]. Lakshmikuttyamma et al. [13] reported increased calpain 2 activity in human CRC. The calpain's role in carcinogenesis and tumor progression has yet to be explored.

Oxidative stress caused by increased free radical generation and/or a decreased antioxidant level in target cells suggested playing an important role in carcinogenesis [14]. Prime targets of free radicals are polyunsaturated fatty acids in cell membranes and their interaction results in lipid peroxidation. Nitric oxide (NO) is inorganic free radical gas produced from L-arginine by NO synthases (NOS). Two NOs are constitutively expressed and a third is inducible by immunological stimuli. Nitric oxide released by inducible NO synthase (iNOS) has been shown to be cytostatic/cytotoxic for tumor cells [15]. Ozgonul et al. [16] reported that CRC patients are exposed to oxidative stress, which might play a role in disease pathogenesis. Bakan et al. [17] reported increased NO production and malondialdehyde (MDA) blood levels of GC patients.

Cytokines stimulate cancer cell growth and contributed to locoregional relapse and metastases [18]. Oxidative stress could increase TNF-α release [14]. It is worth noting that TNF-α acts as a mediator of the apoptotic process and has selective cytotoxicity against malignant breast tumor cells, promoting an apoptotic type of cell death in MCF-7 cells [19]. TNF-α had been postulated as a key player in the tumor microenvironment, but had a paradoxical role in disease evolution: It could act both as necrotic or as a promoting factor, e.g. endogenous TNF-α chronically produced in a tumor microenvironment enhanced both tumor development and spread-

ing, while local administration of high-doses of TNF-α had antiangiogenic and anti-tumoral effects [20].

The present study was designed to investigate plasma levels of sFas and bcl-2 as antiapoptotic indices. Calpain I and II activities, which act as apoptosis upstream regulators, would also be investigated. Recent studies have suggested the important role of oxidative stress in gastric-colon tumors, where they seemed to be involved in the release of proteases and TNF-α. Therefore, CD, LPER, NO and TNF-α plasma levels would be measured. The levels of these indices would be correlated with each other and tumor progression. Moreover, since proteases, i.e. CD and calpain I and II, were also involved in angiogenesis, the balance between apoptosis and angiogenesis would be discussed.

Materials and Methods

Thirty newly diagnosed malignant gastric-colon tumor patients confirmed by pathological examination (14 men, 16 women) with an age range from 6-75 years (mean+/-SD, 46.93±18.92 years) were recruited in this cross-sectional study from a surgical clinic, South Egypt Cancer Institute, Assiut, Egypt between February 2010 and June 2011. As control groups, 30 subjects with benign GIT masses (18 men, 12 women) with an age range from 13-73 years (40.67±18.46 years) served as pathological controls, and 30 healthy volunteers (14 men, 16 women) with an age range from 12-66 years (44.13±14.82 years) serving as healthy controls, selected from screening clinics, were recruited. Patients were enrolled before surgery, radiotherapy and chemotherapy. Demographic, clinical and ultrasonography data were obtained. An upper endoscopy was performed on 10 patients with gastric masses to evaluate the size, site of the lesion and non-tumorous part of the stomach, and to take a biopsy. A colonoscopy was performed on 20 patients with colorectal masses and 15 patients with benign colorectal lesions to evaluate the site, size and numbers of lesions, non-tumorous part of the colon and rectum, and to take a biopsy. Patients with primary malignant tumors in other organs, or a previous malignancy treatment history, or whose tumor tissues were not available, were excluded. Healthy controls' selection criteria included no history of cancer or benign GIT diseases and matching to a patient's age and sex. All patients signed informed consent and the local Medical Ethics Committee approved the study in accordance with the Helsinki Declaration.

For the patients with gastric tumors, a distal subtotal gastrectomy was performed in 7 patients with distal pyloric lesions; a total gastrectomy was performed in two patients (one patient with a mid-body tumor and one patient with a proximal fundal lesion extending to the body), and a proximal subtotal gastrectomy was performed in one patient with proximal fundal lesions. In patients with colorectal tumors, ten patients had rectal cancer; an abdomino-perineal resection was performed in 7 patients, and an anterior resection with restoration of the continuity was performed in 3 patients. An anterior resection with restoration of the continuity was performed in 4 patients with sigmoidal cancer. A left hemicolectomy was performed in 2 patients with left colonic cancer. A right hemi-colectomy was performed in 3 patients with right colonic cancer. An extended right hemicolectomy was performed in 1 patient with transverse colonic cancer. Tissue samples were obtained at diagnosis time and sent for pathological evaluation. Histopathological examination showed that 13 (43.30%) cancer patients had adenocarcinoma, 11 (36.70%) had mucoid carcinoma and 6 (20%) had invasive gastric carcinoma. According to TNM cancer staging, 5 cases (16.60%) were classified as stage I, 9 (30%) as stage II, 10 (33.30%) as stage III and 6 (20%) as stage IV. Of 30 patients with benign GIT masses, 9 (30%) had chronic non-specific inflammation, 6 (20%) had a mesentric cyst, 6 (20%) had Peutz Goiger, 6 (20%) had Juvenile rectal polypi and 3 (10%) had chronic pancreatitis.

Venous blood samples (6 mL) were collected on day of surgery and before any therapeutic administration or surgical intervention, using ethylene-diaminetetraacetic acid as anticoagulant, and plasma was separated by centrifugation at 3000 rpm for 10 min and stored at -20°C. An ELISA kit was used to measure plasma levels of sFas (Code #5251, Medical & Biological Laboratories Co Ltd, Tokyo, Japan) with sensitivity 0.5 ng/ml, bcl-2 (Cat # QIA23, Calbiochem, Darmstadt, Germany) with

sensitivity <1 U/ml and TNF-α (Cat #SK00109-01, Beijing Adipobiotech Santa Clara, CA, USA) with sensitivity 4 pg/ml. Cathepsin D proteolytic activity was assayed, as described by Barrett [21]. A calpain assay was done, as did Yoshimura et al. [22], using Hammerstein- grade casein as substrate. LPER was measured, as described by Grau et al. [23]. The nitric oxide level was determined as total nitrite after deproteinization with ZnSO4 (30%), and color developed by a reaction with Griess reagent was recorded at 550 nm against reagent blank using sodium nitrite 10-100uM as standard [24].

Statistical Analysis

Data were represented as a mean+/-standard deviation or number (%), as appropriate. One-way analysis of variance (ANOVA) and student "t" tests were used to determine statistical differences between groups, as appropriate. Differences in percentage were calculated with a Chi-square test. A P <0.05 was considered statistically significant. A person's correlation was done between parametric variables. Statistical analysis was performed with SPSS software version 16.

Results

Demographic characteristics of all participants as well as imaging and histopathology findings and stages of masses were shown in (Table 1).

sFas, bcl-2, CD, calpain I, calpain II, NO, and TNF-α levels were significantly higher in malignant and benign gastric-colon masses than healthy controls, and in malignant than benign masses. LPER was significantly elevated in malignant than benign masses and healthy controls (Table 2).

In stage IV, plasma levels of sFas, bcl-2 and TNFα were significantly elevated, compared to stages II and III; meanwhile, CD, calpain I, calpain II, LPER and NO were significantly elevated, compared to stage II (Table 3).

sFas, bcl-2, calpain I, calpain II, CD, TNF-α, LPER, and NO levels were significantly elevated in CRC, GC and benign GIT masses than healthy controls, and in CRC and GC than benign GIT masses. In cases of GC, the level of calpain I, calpain II, CD, LPER, and NO were significantly higher than CRC (Table 4).

In healthy controls, significant positive correlations were found between bcl-2 with TNF-α, LPER; between LPER with CD, calpain I; between CD with calpain I; and between NO with calpain II. In patients with benign and malignant GIT masses, positive correlations were found between sFas, bcl-2, CD, calpain I, calpain II, NO, LPER and TNF- α (Table 5).

Discussion

The apoptosis-related genes are expressed in various frequencies in different cancers, and general patterns of activation or inactivation of these genes in malignant tumors cannot be defined. The Fas/FasL system exerts a central role in the apoptosis process. Although there are some studies indicating that gastric carcinomas express higher levels of FasL and lower levels of Fas to evade killing effects of the host immune system [25], there are only few reports addressing their soluble forms. In this study, the sFas plasma level was significantly elevated in patients with CRC and GC, compared to healthy controls and benign GIT masses. The sFas level was significantly elevated in stage IV, compared to stages II and III. Serum sFas levels reported being elevated in patients with locally advanced and metastatic CRC [26]. Liang et al. [7] represented a higher sFas serum level in all GC patients, compared to non-tumoral individuals. Yatsuya et al. [6] reported an elevated sFas serum level only between female GC patients than controls. To explain decreases in Fas and increases in sFas serum levels in GC, the authors hypothesize that translational processing of the Fas gene in gastric tumoral cells might be deranged, leading to production of mostly soluble, rather than membranous, Fas [25]. To confirm this idea, a group of non-tumoral cases were evaluated for sFas plasma levels. Analysis showed a significant difference between sFas levels in non-cancerous and cancer groups, in line with the findings of Li et al. [27]. Tamakoshi et al. [28] suggested that serum sFas can detect people at high risk for cancer prior to diagnosis. Further studies that investigate both soluble and membranous isoforms of the Fas gene may provide valuable information about correlations between the plasma level and tissue expression of Fas gene products, helping in gaining a better understanding of

Table 1. Imaging and histopathology of benign and malignant masses.

Parameters	Benign masses (n= 30)	Malignant tumors (n= 30)
Abdominal ultrasound		
Normal	15 (50.00%)	14 (46.7%)
Mass in right iliac fossa Mass in left iliac fossa	-	3 (10.00%) 9 (30.00%)
Mass in epigastric region	-	3 (10.00%)
Liver enlargement	3 (10.00%)	1 (3.30%)
Cystic swelling related to intestine	6 (20.00%)	-
Dilated intestinal loops with Pseudo kidney appearance	6 (20.00%)	-
Location of masses		
Gastric cancers Distal pyloric lesion Proximal fundal lesion Mid body lesion	-	10 (33.30%) 7(23.33%) 2 (6.67%) 1 (3.33%)
Colorectal cancers Rectum Sigmoid colon Left colon Right colon Transverse colon	-	20 (67.70%) 10 (33.33%) 4(13.33%) 2 (6.67%) 3 (10.00%) 1(3.33%)
Polyps	15 (50.00%)	-
Cysts	9 (30.00%)	-
Tongue growth	3 (10.00%)	-
Parotid swelling	3 (10.00%)	-
Pathology		
Adenocarcinoma	-	13 (43.30%)
Mucoid carcinoma	-	11 (36.70%)
Invasive gastric carcinoma	-	6 (20.00%)
Chronic non specific inflammation	9 (30.00%)	-
Mesentric cyst	6 (20.00%)	-
Peutz Goiger	6 (20.00%)	-
Juvenile rectal polypi	6 (20.00%)	-
Chronic pancreatitis	3 (10.00%)	-
TNM staging		
Gastric		-
Stage II		5 (16.67%) [T2 N1 M0]
Stage III		5 (16.67%) (T3 N1 M0]
Colorectal		
Stage I		5 (16.67%) [T2 N0 M0]
Stage II		4 (13.33%) [T3 N0 M0]
Stage III		5 (16.67%) [T2 N2 M0]
Stage IV		6 (20.00%) [T4 T3M1]

Data are expressed as number (%); TNM, Tumor, Nodes, and Metastases.

Table 2. Comparison of different measured parameters of different studied groups.

Parameters	Healthy controls (n= 30)	Benign masses (n= 30)	Malignant tumors (n= 30)
age (years)	44.13±14.82 12.00-66.00	40.67±18.46 13.00-73.00	46.93±18.92 6.00-75.00
Significance		*P =0.445	*P =0.930 **P =0.395
Gender			
Male	14 (46.70%)	18 (60.00%)	14 (46.70%)
Female	16 (53.30%)	12 (40.00%)	16 (53.30%)
Tumor size (cm^2)	-	9.30±12.82 1.00-36.00	25.40±43.41 10.00-210.00
Significance			***P <0.003***
sFas (ng/ml)	1.39±0.36 0.85-2.10	2.64±0.27 2.20-3.00	9.25±2.69 3.90-13.50
Significance		***P <0.003***	***P <0.0001*** ***P <0.0001***
Bcl-2 (u/ml)	77.75±10.77 55.60-95.80	131.32±16.00 89.30-151.10	245.98±76.05 63.40-381-50
Significance		***P <0.000***	***P <0.0001*** ***P <0.0001***
Cathepsin D (nmol/L)	7.54±1.45 5.20-10.10	18.48±3.33 13.60-25.60	23.44±7.01 11.80-34.50
Significance		***P <0.0001***	***P <0.0001*** ***P <0.0001***
Calpain I (u/ml)	3.82±0.84 2.10-5.30	6.83±0.89 5.00-8.20	9.27±2.49 5.00-13.20
Significance		***P <0.0001***	***P <0.0001*** ***P <0.0001***
Calpain II (u/ml)	7.31±1.60 4.20-9.80	13.76±2.08 10.80-17.10	18.75±4.88 11.00-26.40
Significance		***P <0.0001***	***P <0.0001*** ***P <0.0001***
Lipid peroxide (umol/L)	3.34±0.71 1.90-4.60	3.99±0.43 3.40-4.90	12.00±2.87 6.30-16.40
Significance		***P <0.149***	***P <0.0001*** ***P <0.000***
Nitric oxide (umol/L)	5.16±1.36 2.50-8.10	7.09±1.26 4.80-8.80	15.67±4.60 8.80-23.60
Significance		***P <0.010***	***P <0.0001*** ***P <0.0001***
Tumor necrosis factor-α (pg/ml)	89.58±10.60 7.80-105.40	146.75±25.20 110.40-182.30	309.10±89.86 194.10-493.80
Significance		***P <0.0001***	***P <0.0001*** ***P <0.0001***

Data are expressed as mean+/-standard deviation (range) or number (%) as appropriate.

Table 3. Comparison of measured parameters between subgroups of malignant gastrointestinal tract cancers according to TNM tumor stages.

Variables	**Tumor Stage**			
	Stage I (n = 5, 16.67%)	**Stage II** (n = 9, 30.00%)	**Stage III** (n = 10, 33.33%)	**Stage IV** (n = 6, 20.00%)
sFas (ng/ml)	9.84±2.63	8.11±1.93	8.66±2.36	11.47±3.32
Significance		**P =0.226*	**P =0.396* ***P =0.637*	**P =0.292* *****P <0.017*** ******P <0.039***
Bcl-2 (u/ml)	265.16±82.19	206.64±36.24	230.79±77.88	314.32±76.85
Significance		**P <0.137*	**P <0.367* ***P <0.449*	**P <0.246* *****P <0.006*** ******P <0.026***
Cathepsin D (nmol/L)	23.28±6.12	19.54±5.58	24.29±7.91	27.98±6.22
Significance		**P =0.324*	**P =0.784* ***P =0.133*	**P =0.255* *****P <0.024*** ****P =0.293*
Calpain I (u/ml)	8.92±1.70	7.94±2.11	9.70±2.85	10.85±2.30
Significance		**P =0.468*	**P =0.554* ***P =0.120*	**P =0.191* *****P <0.028*** ****P =0.357*
Calpain II (u/ml)	17.86±4.21	16.29±4.01	19.60±5.17	21.78±5.06
Significance		**P =0.552*	**P =0.503* ***P =0.135*	**P =0.178* *****P <0.035*** ****P =0.374*
Lipid peroxide (umol/L)	12.00±2.06	10.60±2.17	11.84±3.11	14.38±3.03
Significance		**P =0.358*	**P =0.914* ***P =0.324*	**P =0.154* *****P <0.013*** ****P =0.078*
Nitric oxide (umol/L)	16.12±2.89	13.50±3.75	15.45±5.11	18.90±5.00
Significance		**P =0.297*	**P =0.784* ***P =0.345*	**P <0.308* *****P <0.028*** ****P =0.142*
Tumor necrosis factor-α (pg/ml)	338.54±103.98	255.40±53.37	287.80±66.49	400.63±93.04
Significance		**P =0.060*	**P =0.232* ***P =0.360*	**P =0.187* *****P <0.001*** ******P <0.008***

Data are expressed as mean +/- standard deviation. * P: significance versus stage I; ** P: significance versus stage II; ***P: significance versus stage III.

Table 4. Comparison of different measured biochemical parameters of different studied subgroups.

Variables	Healthy controls (n= 30)	Benign masses (n= 30)	Colorectal tumors (n= 20)	Gastric tumors (n= 10)
sFas (ng/ml)	1.39±0.36 0.85-2.10	2.64±0.27 2.20-3.00	9.11±2.85 3.90-13.50	9.54±2.42 4.90-13.10
Significance		***P <0.003**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P =0.483*
Bcl-2 (u/ml)	77.75±10.77 55.60-95.60	131.32±15.98 89.30-151.10	245.41±76.86 152.40-381.50	247.13±78.51 63.40-311.40
Significance		***P <0.0001**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P =0.922*
Cathepsin D (nmol/L)	7.54±1.45 5.20-10.10	18.48±3.33 13.80-25.60	22.26±6.79 13.20-32.10	25.79±7.18 11.80-32.50
Significance		***P <0.0001**	***P <0.0001** ****P <0.004**	***P <0.0001** ****P <0.0001** *****P <0.045**
Calpain I (u/ml)	3.82±0.84 2.10-5.30	6.83±0.89 5.00-8.20	8.80±2.32 5.30-12.50	10.22±2.68 5.00-13.20
Significance		***P <0.003**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P <0.021**
Calpain II (u/ml)	7.31±1.60 4.20-9.80	13.76±2.08 10.80-17.10	17.70±4.82 11.00-26.40	20.86±4.48 11.00-25.10
Significance		***P <0.003**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P <0.010**
Lipid peroxide (umol/L)	3.34±0.71 1.90-4.60	3.99±0.43 3.40-4.90	11.52±2.85 6.80-16.40	12.97±2.82 6.30-15.70
Significance		*P =0.138	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P <0.029**
Nitric oxide (umol/L)	5.16±1.36 2.50-8.10	7.09±1.26 4.80-8.90	14.94±4.33 9.20-23.60	17.12±5.00 8.80-21.60
Significance		***P <0.009**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P <0.049**
Tumor necrosis factor-α (pg/ml)	89.58±10.60 71.80-105.40	146.75±25.20 110.40-182.30	308.24±97.34 194.10-493.80	310.83±77.56 196.20-405.20
Significance		***P <0.0001**	***P <0.0001** ****P <0.0001**	***P <0.0001** ****P <0.0001** *****P =0.903*

Data are expressed as mean+/-standard deviation (range);*P: significance versus controls; **P: significance versus benign tumors; ***P: significance versus colorectal tumor.

Table 5. Correlation between different measured parameters in different studied groups.

Parameters	sFas	Bcl-2	Tumor necrosis factor-α	Lipid peroxide	Cathepsin D	Calpain I	Calpain II
Bcl-2 (u/ml)							
Controls	0.036 (0.849)						
Benign	**0.786(0.0001)**						
Malignant	**0.941 (0.0001)**						
Tumor necrosis factor-α (pg/ml)							
Controls	0.000(0.999)	**0.860(0.0001)**					
Benign	**0.868(0.0001)**	**0.757(0.0001)**					
Malignant	**0.872(0.0001)**	**0.871(0.0001)**					
Lipid peroxide (umol/L)							
Controls	0.095(0.617)	**0.485(0.007)**	0.304(0.103)				
Benign	**0.777(0.0001)**	**0.544(0.002)**	**0.865(0.0001)**				
Malignant	**0.915(0.0001)**	**0.891(0.0001)**	**0.820(0.0001)**				
Cathepsin D (nmol/L)							
Controls	0.129(0.497)	0.092(0.629)	-0.120(0.529)	**0.456(0.011)**			
Benign	**0.592(0.001)**	**0.726(0.0001)**	**0.720(0.0001)**	**0.759(0.0001)**			
Malignant	**0.815(0.0001)**	**0.795(0.0001)**	**0.668(0.0001)**	**0.913(0.0001)**			
Calpain I (u/ml)							
Controls	0.019(0.922)	0.145(0.443)	0.037(0.847)	**0.415(0.023)**	**0.686(0.0001)**		
Benign	**0.588(0.001)**	**0.789(0.0001)**	**0.639(0.0001)**	**0.548(0.002)**	**0.903(0.0001)**		
Malignant	**0.771(0.0001)**	**0.765(0.0001)**	**0.601(0.0001)**	**0.874(0.0001)**	**0.980(0.0001)**		
Calpain II (u/ml)							
Controls	0.119(0.532)	0.177(0.349)	0.051(0.790)	0.034(0.860)	0.192(0.311)	0.254(0.175)	
Benign	**0.476(0.008)**	**0.674(0.0001)**	**0.553(0.002)**	**0.548(0.002)**	**0.860(0.0001)**	**0.845(0.0001)**	
Malignant	**0.754(0.0001)**	**0.727(0.0001)**	**0.611(0.0001)**	**0.861(0.0001)**	**0.970(0.0001)**	**0.981(0.0001)**	
Nitric oxide (umol/L)							
Controls	0.196 (0.298)	-0.162(0.392)	-0.056(0.769)	-0.254(0.175)	0.218(0.248)	0.356 (0.053)	**0.568(0.001)**
Benign	**0.374(0.040)**	**0.667(0.0001)**	**0.392(0.032)**	**0.315(0.090)**	**0.790(0.001)**	**0.861(0.0001)**	**0.870(0.0001)**
Malignant	**0.675(0.0001)**	**0.659(0.0001)**	**0.553(0.002)**	**0.737(0.0001)**	**0.877(0.0001)**	**0.911(0.0001)**	**0.914(0.0001)**

DOI: 10.5455/aces.20120216121853
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the molecular basis of changes, and may introduce sFas as a useful and non-invasive biomarker for early detection of GC and CRC.

In this study, the bcl-2 plasma level was significantly elevated in GC and CRC patients, compared to healthy controls and benign GIT masses. Konturek et al. [29] reported that bcl-2 expression was detected at a protein level (western blot) in the majority of GC samples. Lauwers et al. [30] reported that in 72% of immunostained GC samples, there was positive staining for bcl-2. Thus, inhibition of apoptosis via increased bcl-2 expression appeared to contribute to cancer cell growth. Bcl-2 expression had been detected previously in colon tumors [31].

Cancer development is characterized by disarrangement of intra- and extracellular metabolism. Cancer cells overexpress and secrete lysosomal proteases-cathepsins. CD, secreted as a proenzyme, requires a more acidic pH to be proteolytically active. Extracellular pH in tumors is more acidic than normal tissues. Excessive CD accumulates in cancer cells and the proenzyme is hyper-secreted in a tumor micro-environment [32]. In this study, CD levels were significantly elevated in patients with malignant tumors versus benign masses and healthy controls. CD activity was more in patients with grade IV, compared to grade II. Meanwhile, the CD level was significantly elevated in GC versus CRC, indicating that the proteolytic effect is more extensive in GC. CD would be able to digest many types of proteins from ECM, thus providing nutrients, amino acid, and space for invasive cancer cells. Also, CD induced angiogenesis and contributed to malignant phenotypes by inducing tumor cell migration, nodal growth and metastasis [33]. The abnormal cytoplasmic staining pattern of CD reflects invasive potential of GC cells [33]. Even in early GC, CD facilitates an early phase of tumor progression, such as cell proliferation and local dissemination [34]. ROS causes oxidative damage of cell membranes in cancer results in the increase of membrane permeability. That implies an influx of CD to extracellular fluid. Therefore, elevated activity of CD is observed in plasma as an evidence for impairment of general cellular functions, like maintenance of the barrier between intracellular and extracellular environments [11]. This is further supported by a significant positive correlation between CD and LPER. Also, it had been reported that cathepsin came from two kinds of cells: carcinomatous and inflammatory cells that constitute an immunologic response to neoplasm [35].

In this study, calpain I and calpain II levels were significantly elevated in patients with malignant than benign GIT masses and healthy controls, and in GC versus CRC. In gastric-colon tumors, calpain I and calpain II levels were significantly elevated in stage IV, compared to stage II. Lakshmikuttyamma et al. [13] reported that activity and protein expression of calpain II was significantly higher in colorectal adenocarcinomas than healthy controls. Protein expression of calpain II was 2- to 3- fold greater, while enzyme activity increased only ~1.5-fold in the colonic tumor. A marked increase in calpain II staining in immunohistochemical studies indicated that elevated calpain levels in colon cancer were due to increased enzyme production rather than an alteration in enzyme conformation [13]. The role of calpains in colon cancer development had not been postulated until today. Proto-oncogenes c-fos and c-jun, several cytoskeletal proteins, tumor suppressor protein p53, and signaling molecules protein kinase C and focal adhesion kinase were substrates for calpain. Calpain mediated focal adhesion kinase cleavage, and disassemblies accompany v-Src-induced morphologic transformation [36]. In response to v-Src activation, Carragher et al. [36] noted an increase in total protein levels of calpain II and decreased levels of calpastatin in chicken embryo fibroblasts. Elevated expression of calpain II in CRC may act on p53 and may be followed by a decrease in apoptosis. Likewise, the calpain mediated cleavage of Bax promotes the proapoptotic effect of Bax [37], and the calpain cleavage of pro-caspase-7 and pro-caspase-3 leads to activation of these proteases [38]. Cross-talk between calpain and caspases seems to be important for apoptosis regulation in colon tumors.

It has been suggested that active oxygen species generated in inflamed tissues could damage target cells, resulting in DNA damage, and contribute to tumor development. Nitric oxide is known, together with other ROS, to induce cytotoxicity and cytostasis [39]. Various studies showed that NOS activity and NO synthesis were high in tumor tissue and in plasma [14]. In this study, NO and LEPR levels were significantly elevated in malignant gastric-colon masses than healthy controls and also in gastric than colorectal cancer. Meanwhile, NO was higher in patients with malignant than benign masses. Lipid peroxides and NO plasma levels were higher in patients with gastric-colon tumor stage IV than stage II. Changes in the oxidative-antioxidative system in CRC were reported in earlier studies [35]. Haklar et al. [14] reported increased NO in tissues of colon tumors. Others reported an increase in MDA and NO plasma levels in GC [14, 17]. NO can regulate vascular endothelial growth factor (VEGF) roles in inducing angiogenesis by stimulating vascular endothelial cell proliferation and migration. The vascular endothelial growth factor can increase iNOS activity [40]. iNOS is highly expressed in many human cancers, such as colon, stomach, ovaries, breast, etc. [41]. Low concentration of NO is known to maintain gastric mucosal integrity, inhibit adhesion molecule expression, cytokine and chemokine synthesis, and leukocyte adhesion and transmigration. On the contrary, excessive and prolonged NO production promote tumor growth through creating neovasculature [42]. Some studies reported high lipid peroxidation in human CRC tissue [35] and GC [43]. In addition to inducing DNA, lipid, and protein damage, oxidative damage to protein-coding or non-coding RNA may potentially cause errors in protein synthesis or dysregulation of gene expression [44].

TNF-α is responsible for several immunologic functions. The TNF-α level is often increased in cancer patients and it may promote tumor growth and invasion [20]. In this study, the TNF-α level was significantly elevated in patients with benign and malignant masses than healthy controls, in malignant versus benign masses, and in cancer patients with stage IV than stages II and III. Others [45, 46] reported significant elevation in the TNF-α serum level in CRC patients.

In conclusion, the present study clarified that patients with gastric-colon malignancy exhibited decreased apoptosis, as evident by the significant increase in anti-apoptotic indices, i.e. sFas and bcl-2, and increased angiogenic activity, as evident by enhanced proteolytic activity of cathepsin-D, calpain I and II. The plasma levels of LPER, NO as well as TNF-α, which indicate angiogenic, oxidative stress and inflammatory responses, were also increased. The levels of these indices were higher in gastric than colorectal cancers, reflecting the more aggressive behavior of the earlier. Thus, decreased apoptosis and enhanced angiogenesis give growth priority to gastric-colon tumors. Therefore, analysis of these markers may help in studying tumor aggressiveness, and predicting their behavior and blockage of angiogenic factors may delay a tumor's spread.

Competing interest

The authors declare that they have no conflict of interest.

References

1. Bertuccio P, Chatenoud L, Levi F, Praud D, Ferlay J, Negri E, et al. Recent patterns in gastric cancer: a global overview. Int J Cancer 2009;125:666-673.
2. Stewart BW, Kleihues P. World cancer report. In: Stewart BW, Kleihues P, (eds). Colorectal Cancer, IARC Press, Lyon, 2003.
3. Abbott DE, Margaryan NV, Jeruss JS, Khan S, Kaklamani V, Winchester DJ, et al. Reevaluating cathepsin D as a biomarker for breast cancer: serum activity levels versus histopathology. Cancer Biol Ther 2010;9:23-30.
4. Thorburn A. Death receptor-induced cell killing. Cell Signal. 2004;16:139-144.

5. Nahta R, Esteva FJ. Bcl-2 antisense oligonucleotides: a potential novel strategy for the treatment of breast cancer. Semin Oncol 2003;30(5 Suppl 16):143-149.

6. Yatsuya H, Toyoshima H, Tamakoshi K, Tamakoshi A, Kondo T, Hayakawa N, et al. JACC Study Group. Serum levels of insulin-like growth factor I, II, and binding protein 3, transforming growth factor beta-1, soluble fas ligand and superoxide dismutase activity in stomach cancer cases and their controls in the JACC Study. J Epidemiol 2005;15 Suppl 2:S120-125.

7. Liang QL, Pan DC, Yin ZM, Liu GX, Yang Q, Xie JR, et al. [Clinical value of serum soluble Apo-1/Fas for predicting biological behaviors and prognosis of gastric carcinoma]. [Article in Chinese]. Ai Zheng 2002;21:174-176.

8. Schneider HJ, Sampson SA, Cunningham D, Norman AR, Andreyev HJ, Tilsed JV, et al. Bcl-2 expression and response to chemotherapy in colorectal adenocarcinomas. Br J Cancer 1997;75:427-431.

9. Giannoulis K, Fountzilas G, Angouridakis N, Angouridaki C, Giannoulis E, Gamvros O. Serum levels of bcl-2 in patients with colorectal cancer. Tech Coloproctol 2004;8 Suppl 1:s56-58.

10. Dragutinović V, Izrael-Zivković L, Radovanović N. Relation of matrix metalloproteinase-9 to different stages of tumors in the serum of gastric cancer. Dig Dis Sci 2009;54:1203-1207.

11. Skrzydlewska E, Sulkowska M, Wincewicz A, Koda M, Sulkowski S. Evaluation of serum cathepsin B and D in relation to clinicopathological staging of colorectal cancer. World J Gastroenterol 2005;11:4225-4229.

12. Carragher NO, Westhoff MA, Riley D, Potter DA, Dutt P, Elce JS, et al. v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. Mol Cell Biol 2002;22:257-269.

13. Lakshmikuttyamma A, Selvakumar P, Kanthan R, Kanthan SC, Sharma RK. Overexpression of m-calpain in human colorectal adenocarcinomas. Cancer Epidemiol Biomarkers Prev 2004;13:1604-1609.

14. Haklar G, Sayin-Ozveri E, Yüksel M, Aktan AO, Yalçin AS. Different kinds of reactive oxygen and nitrogen species were detected in colon and breast tumors. Cancer Lett 2001;165:219-224.

15. Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, del Soldato P. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. Proc Natl Acad Sci USA 2001;98:4202-4208.

16. Ozgonul A, Aksoy N, Dilmec F, Uzunkoy A, Aksoy S. Measurement of total antioxidant response in colorectal cancer using a novel automated method. Turk J Med Sci 2009; 39:503–506.

17. Bakan E, Taysi S, Polat MF, Dalga S, Umudum Z, Bakan N, et al. Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. Jpn J Clin Oncol 2002;32:162-166.

18. Kozłowski L, Zakrzewska I, Tokajuk P, Wojtukiewicz MZ. Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. Rocz Akad Med Bialymst 2003;48:82-84.

19. Donato NJ, Klostergaard J. Distinct stress and cell destruction pathways are engaged by TNF and ceramide during apoptosis of MCF-7 cells. Exp Cell Res 2004;294:523-533.

20. Balkwill F. Tumor necrosis factor or tumor promoting factor? Cytokine Growth Factor Rev 2002;13:135-141.

21. Barrett AJ. Cathepsin D. Purification of isoenzymes from human and chicken liver. Biochem J 1970;117:601-607.

22. Yoshimura N, Kikuchi T, Sasaki T, Kitahara A, Hatanaka M, Murachi T. Two distinct Ca2+ proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. J Biol Chem 1983;258:8883-8889.

23. Grau A, Codony R, Rafecas M, Barroeta AC, Guardiola F. Lipid hydroperoxide determination in dark chicken meat through a ferrous oxidation-xylenol orange method. J Agric Food Chem 2000;48:4136-4143.

24. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988;141:2407-2412.

25. Li Z, Wang Z, Zhao Z, Zhang Y, Ke Y. [Expression of Fas, FasL and IFN-gamma in gastric cancer]. [Article in Chinese]. Beijing Da Xue Xue Bao 2003;35:386-389.

26. Kushlinskii NE, Britvin TA, Abbasova SG, Perevoshchikov AG, Prorokov VV, Kostanyan IA, et al. Soluble Fas antigen in the serum of patients with colon cancer. Bull Exp Biol Med 2001;131:361-363.

27. Li H, Liu N, Guo L, Li JW, Liu J. Frequent expression of soluble Fas and Fas ligand in Chinese stomach cancer and its preneoplastic lesions. Int J Mol Med 2000;5:473-476.

28. Tamakoshi A, Nakachi K, Ito Y, Lin Y, Yagyu K, Kikuchi S, et al. Japan Collaborative Cohort Study Group. Soluble Fas level and cancer mortality: findings from a nested case-control study within a large-scale prospective study. Int J Cancer 2008;123:1913-1916.

29. Konturek PC, Konturek SJ, Sulekova Z, Meixner H, Bielanski W, Starzynska T, et al. Expression of hepatocyte growth factor, transforming growth factor alpha, apoptosis related proteins Bax and Bcl-2, and gastrin in human gastric cancer. Aliment Pharmacol Ther 2001;15:989-999.

30. Lauwers GY, Scott GV, Karpeh MS. Immunohistochemical evaluation of bcl-2 protein expression in gastric adenocarcinomas. Cancer 1995;75:2209-2213.

31. Baretton GB, Diebold J, Christoforis G, Vogt M, Müller C, Dopfer K, et al. Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of carcinogenesis and prognostic significance. Cancer 1996;77:255-264.

32. Zaidi N, Maurer A, Nieke S, Kalbacher H. Cathepsin D: a cellular roadmap. Biochem Biophys Res Commun 2008;376:5-9.

33. Ikeguchi M, Fukuda K, Oka S, Hisamitsu K, Katano K, Tsujitani S, et al. Micro-lymph node metastasis and its correlation with cathepsin D expression in early gastric cancer. J Surg Oncol 2001;77:188-194.

34. Leto G, Tumminello FM, Crescimanno M, Flandina C, Gebbia N. Cathepsin D expression levels in non-gynecological solid tumors: clinical and therapeutic implications. Clin Exp Metastasis 2004;21:91-106.

35. Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga-Koda L, Sulkowska M. Lipid peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol 2005;11:403-406.

36. Carragher NO, Fincham VJ, Riley D, Frame MC. Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J Biol Chem 2001;276:4270-4275.

37. Toyota H, Yanase N, Yoshimoto T, Moriyama M, Sudo T, Mizuguchi J. Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. Cancer Lett 2003;189:221-230.

38. Blomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, et al. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis"? J Biol Chem 2001;276:10191-10198.

39. Tamir S, Tannenbaum SR. The role of nitric oxide (NO.) in the carcinogenic process. Biochim Biophys Acta 1996;1288:F31-36.

40. Lee JC, Chow NH, Wang ST, Huang SM. Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. Eur J Cancer 2000;36:748-753.

41. Chen T, Nines RG, Peschke SM, Kresty LA, Stoner GD. Chemopreventive effects of a selective nitric oxide synthase inhibitor on carcinogen-induced rat esophageal tumorigenesis. Cancer Res 2004;64:3714-3717.

42. Ilhan N, Ilhan N, Ilhan Y, Akbulut H, Kucuksu M. C-reactive protein, procalcitonin, interleukin-6, vascular endothelial growth factor and oxidative metabolites in diagnosis of infection and staging in patients with gastric cancer. World J Gastroenterol 2004;10:1115-1120.

43. Farinati F, Cardin R, Degan P, Rugge M, Mario FD, Bonvicini P, et al. Oxidative DNA damage accumulation in gastric carcinogenesis. Gut 1998;42:351-356.

44. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol 2010;38:96-109.

45. Kamińska J, Kowalska MM, Nowacki MP, Chwaliński MG, Rysińska A, Fuksiewicz M. CRP, TNF-alpha, IL-1ra, IL-6, IL-8 and IL-10 in blood serum of colorectal cancer patients. Pathol Oncol Res 2000;6:38-41.

46. Szkaradkiewicz A, Marciniak R, Chudzicka-Strugała I, Wasilewska A, Drews M, Majewski P, et al. Proinflammatory cytokines and IL-10 in inflammatory bowel disease and colorectal cancer patients. Arch Immunol Ther Exp (Warsz) 2009;57:291-294.