Cathepsin B expression in colorectal cancer in a Middle East population: Potential value as a tumor biomarker for late disease stages

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Abstract. Cathepsin B (CTSB), is a cysteine protease belonging to the cathepsin (Clan CA) family. The diagnostic and prognostic significance of increased CTSB in the serum of cancer patients have been evaluated for some tumor types. CTSB serum and protein levels have also been reported previously in colorectal cancer (CRC) with contradictory results. The aim of the present study was to investigate CTSB expression in CRC patients and the association of CTSB expression with various tumor stages in a Middle East population. Serum CTSB levels were evaluated in 70 patients and 20 healthy control subjects using enzyme-linked immunosorbant assay (ELISA) technique. CTSB expression was determined in 100 pairs of CRC tumor and adjacent normal colonic tissue using quantitative PCR for mRNA levels. Detection of CTSB protein expression in tissues was carried out using both immunohistochemistry and western blotting techniques. ELISA analysis showed that in sera obtained from CRC patients, the CTSB concentration was significantly higher in late stage patients with lymph node metastases when compared to early stage patients with values of 2.9 and 0.33 ng/ml, respectively (P=0.001). The majority of tumors studied had detectable CTSB protein expression with significant increased positive staining in tumors cells when compared with matched normal colon subjects (P=0.006). The mRNA expression in early stage CRC compared to late stage CRC was 0.04±0.01 and 0.07±0.02, respectively. Increased mRNA expression was more frequently observed in the advanced cancer stages with lymph node metastases when compared with the control (P=0.002). Mann-Whitney test and paired t-test were used to compare serum CTSB and mRNA levels in early and late tumor stage. A subset of four paired tissue extracts were analyzed by western blotting. The result confirmed a consistent increase in the CTSB protein expression level in tumor tissues compared with that noted in the adjacent normal mucosal cells. These findings indicate that CTSB may be an important prognostic biomarker for late stage CRC and cases with lymph node metastases in the Middle Eastern population. Monitoring serum CTSB in CRC patients may predict and/or diagnose cases with lymph node metastases.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant neoplasms worldwide and the third leading cause of cancer-related deaths in patients worldwide. CRC accounts for ~10% of all cancer incidence and mortality (1,2). CRC has the third highest incidence (after breast and lung cancer) and the fourth highest mortality rate (after lung, liver and stomach cancer) (3). In Saudi Arabia, CRC is the second most prevalent malignancy among all ages (10.3%) and is the commonest malignancy in males (11.8%) (4). CRC incidence is increasing in Saudi Arabia and there is an urgent need to define novel predictive/prognostic biomarkers.

CRC develops through multiple steps, with the sequential acquisition of genetic alterations in key tumor suppressors and oncogenes (5). Recently De Rosa *et al* stated that there is an urgent need to define novel predictive/prognostic biomarkers that may allow surgeons and clinical oncologists to choose the appropriate therapy for various patients (6,7).

The cathepsin family of lysosomal proteases has been increasingly recognized for their altered expression in cancer patients and their role in facilitating tumor progression. Cathepsins are a group of multifunctional cysteine and aspartyl

proteases that regulate tumor growth, invasion, migration, metastases and angiogenesis (8). Cathepsin B (CTSB; is the HUGO-approved official gene symbol) primarily functions as an endopeptidase within endolysosomal compartments in normal cells.

However, during malignant transformation, the regulation of CTSB can be altered at multiple levels resulting in overproduction of CTSB which is of significant importance in various pathologies and oncogenic processes (9). Regulation of CTSB can be altered at multiple levels during malignant transformation in tumor cells, or also in adjacent inflammatory cells, resulting in enhanced local release. It has been suggested that CTSB is a promising target for therapy, chemoprevention and molecular detection of neoplasia (10). Activity of CTSB is known to be important for tumorigenesis, angiogenesis, invasion and metastases (11,12).

Increased levels of CTSB have been observed in samples of primary and metastatic tumors in different types of cancer (13-17). In clinical studies elevated levels of CTSB expression have been reported in prostate cancer (18) gliomas (19) melanomas (20), breast cancer (21,22) and lung squamous cell carcinoma (23).

Extracellular CTSB is involved in cell invasion whereas intracellular CTSB may also mediate malignant properties of CRC cells (24). It was recently reported that 82% of CRC patients had tumors that expressed enhanced CTSB (25). Other studies suggested that CTSB expression or activity may peak during early stage cancer and subsequently decline with advanced disease (17,25). In either case, these reports point to a possible role of CTSB in either or both early and late alterations that lead to tumor formation in the colon.

In light of the above studies, and considering important ethnic background differences, we used two strategies in the present study. The first was to investigate CTSB levels in Saudi Arabia patient sera at different tumor stages and the second was to investigate mRNA gene and protein expression in paired cancerous and normal adjacent tissue samples. The present study demonstrated that the serum level of CTSB was significantly higher in late stage CRC compared to early stage as well as higher in tumor samples vs. healthy controls. The results also confirmed that there was a significant increase in the CTSB expression at the mRNA and protein levels in tumor tissue when compared to normal adjacent mucosa in late stage CRC. Our conclusion is that CTSB is a promising biomarker for predicting CRC progression and detecting the late stages of the disease in Middle East populations.

Materials and methods

Clinical samples. Ninety samples of colorectal tumors and adjacent normal tissue (at least 10 cm from each tumor) were obtained from patients undergoing surgical resection between 2013 and 2015 at King Khalid University Hospital, King Saud University Riyadh (KSA). The patient clinicopathological characteristics are shown in Table I. Tissues were collected after obtaining the written informed consent of the patients, according to the protocol approved by the Ethics Committee at King Saud University. Paired tissue samples were frozen in liquid nitrogen within 15 min from resection, and stored at -80˚C until used. Demographic characteristics, including age and gender, tumor site and histological type of CRC were recorded. The histological stage of the tumor was determined in accordance with the Union International Cancer Control (UICC)-TNM staging system. Tumor localization, UICC stage and tumor differentiation (grading) were in accordance with the WHO classification (25), and documented in a database prospectus. Furthermore, and in order to minimize possible influences of radiotherapy and chemotherapy on CTSB status and prognosis, all patients who had undergone neoadjuvant or adjuvant therapy were excluded from this cohort.

Table I. Clinicopathological characteristics of the CRC patients.

Characteristics	No. of patients
Mean age (58 years)	
<58	48
≥58	52
Gender	
Male	54
Female	46
Primary tumor	
Colon	77
Rectum	13
Tumor staging (UICC, 2010)	
I	5
II	39
III	28
IV	28

Different clinicopathological characteristics of the study population. CRC, colorectal cancer; UICC, Union for International Cancer Control.

Enzyme-linked immunosorbent assay (ELISA). A 5-ml blood sample from each participant patient and healthy control subject was allowed to clot for 1 h at room temperature. Each clotted sample was centrifuged at 5,000 rpm for 10 min. All sera were aliquoted and frozen at -80˚C until used. CTSB concentrations in the sera were determined by a human cathepsin B kit using human CTSB ELISA kit (LS-F1026; Life Span Biosciences, Inc., Seattle, WA, USA) according to the manufacturer's instructions. The sera were analyzed in duplicate at a dilution of 1:50.

Sample preparation. Total cellular RNA and protein were extracted according to the manufacturer's instructions using the PARIS™ kit (Ambion, Foster City, CA, USA) from frozen paired tumor and adjacent normal tissue as recommended by the manufacturer.

Preparation of total RNA. Pure, concentrated RNA was eluted in buffer provided as a kit ingredient, and stored at -80˚C for further analysis. The purity of the total RNA was assessed by measuring the A260/280 ratio (1.8-2.0), using an ultraviolet spectrophotometer. To remove the contamination of genomic DNA, RNA samples were treated with RNAse-free DNAase

(Ambion). For cDNA synthesis, the High Capacity cDNA kit was used to perform reverse transcriptase reaction (cat. no. 4368814; Applied Biosystems, Foster City, CA, USA).

Real-time polymerase chain reaction. PCR samples were prepared using Fast SYBR®-Green Master Mix (cat. no. 4385612; Applied Biosystems), and the following CTSB primers (Integrated DNA Technologies, Leuven, Belgium) were used to detect the expression of CTSB by RT-PCR: forward, 5'-CCAGGGAGCAAGACAGAGAC-3' and reverse, 5'-GAGACTGGCGTTCTCCAAAG-3'; and GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCAT GGACTGTGGTCATGA-3'. The experiments were run on a ViiA™ 7 Real-Time PCR system. Results of CTSB mRNA expression were measured relative to GAPDH gene expression using the $2^{-\Delta\Delta Ct}$ method.

Construction of tissue microarrays (TMAs). TMAs were constructed from paraffin-embedded tumor blocks using a manual tissue arrayer (Array Mold kit D; IHC World, Woodstock, MD, USA) as previously described (26). The detection of CTSB expression was performed using streptavidin-biotinylated horseradish peroxidase (S-ABC) kit (NovoLink Max Polymer Detection System; Novocastra, Milton Keynes, UK) according to the manufacturer's instructions. As a negative control, the same procedure was conducted with the omission of the primary antibody. The expression of CTSB in tumor and normal samples was analyzed using the eSlide capture device (ScanScope CS; Aperio Technologies Inc., Vista, CA, USA).

Immunohistochemistry. Immunohistochemistry staining was carried out on 5-μm sections of TMA blocks. The detection of CTSB expression was performed using streptavidin-biotinylated horseradish peroxidase (S-ABC) kit (Novo Link Max Polymer Detection System; Novocastra). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled H_2O for 5 min, and then, the slides were washed in Tris-buffered saline (TBS) for 10 min. Non-specific binding of antibodies was blocked by incubation with protein block (Novocastra) for 5 min. Subsequently, the slides were incubated with human CTSB monoclonal mouse antibody (Abazyme, Cambridge, MA, USA) primary antibody (1:100) for 1 h at room temperature. Slides were washed in 3X TBS for 3 min, and then incubated with biotinylated anti-mouse IgG (Novocastra) for 30 min. Peroxidase was detected using diaminobenzedine (DAB) substrate (Novocastra). Finally, slides were counterstained with Mayer's hematoxylin (Novocastra). As a negative control, the same procedure was conducted with the omission of the primary antibody. The expression of CTSB in tumor and normal samples was analyzed using the eSlide capture device (ScanScope CS).

Image analysis. High-resolution, whole-slide digital scans of all TMA glass slides were created using a ScanScope slide scanner (Aperio Technologies, Inc.) as previously described (26).

Western blotting. Four pairs of CRC tumors and adjacent normal tissue protein samples were isolated from early and late stage CRC using PARIS™ kit. Total protein concentration was determined using Bradford protein reagent (Bio-Rad, Hercules CA, USA). Soluble proteins (20 μg) were loaded on 10% precast TGX gels and were analyzed by immunoblotting with anti-CTSB (dilution 1:100; EMD Millipore, Billerica MA, USA). Reactivity was detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Bio-Rad). Membranes were developed using C-Digit Blot Scanner (LI-COR, Hamburg, Germany).

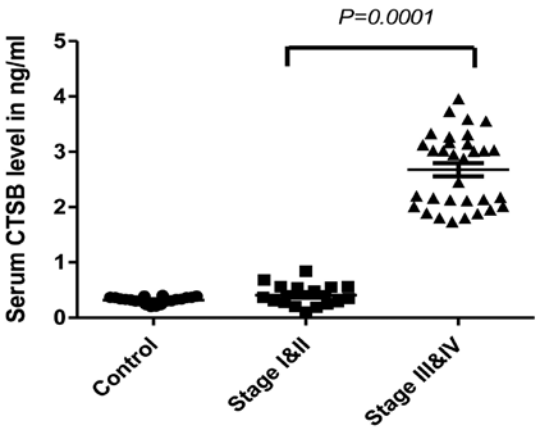

Figure 1. Serum levels of cathepsin B in colorectal cancer patients. CTSB levels from both healthy volunteers (control) and colorectal cancer patients were determined by ELISA. The average levels of CTSB in late stage CRC patients were significantly higher than levels in the control subjects (P=0.0001). Early stage patients showed no difference in regards to CTSB when compared with the controls.

Statistical analysis. Statistical analysis was performed using Graph Pad Prism software (version 5.0). The means between the two groups were compared using Mann-Whitney test and paired t-test in order to compare serum CTSB and mRNA levels in early and late tumor stage. A P-value of ≤0.05 was considered significant.

Ethics statement. The present study was approved by the Ethics Committee of King Khalid University Hospital, King Saud University. Patient consent was obtained for the present study.

Results

Serum cathepsin B levels in CRC patients. To evaluate the CTSB level in sera, two groups of patients were analyzed: early and late stage CRC patients. The level of CTSB was significantly increased in the sera of patients with late stage CRC when compared to the healthy controls. The medians were 2.9 and 0.33 ng/ml, respectively (P<0.0001) (Fig. 1). There was no significant difference between early stage cancer patients and healthy control subjects.

Cathepsin B mRNA is increased in late stage CRC patients. CTSB was analyzed at the mRNA level in a series of human paired specimens at early (stage I and II) and late stage (III and VI) CRC. The relative levels of CTSB mRNA in tumor CRC samples were significantly higher than levels in the

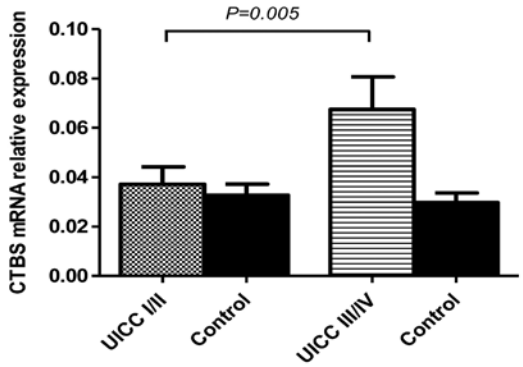

Figure 2. Cathepsin B mRNA expression in colorectal cancer. Relative *CTSB* mRNA levels were determined by q-PCR in human advanced adenomas and adenocarcinomas compared to the paired adjacent healthy tissue. CTSB gene expression showed a statistically significant difference in mRNA expression between early and advanced stages of colorectal cancer (P=0.005).

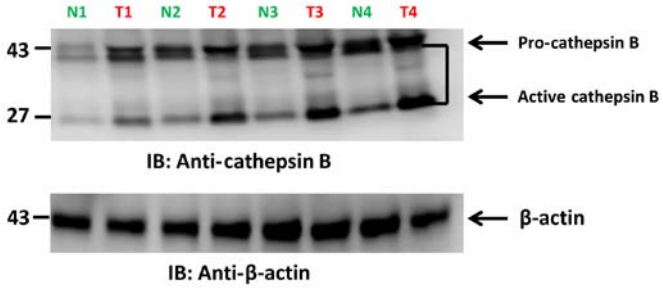

Figure 3. Cathepsin B protein patterns in paired samples of normal human colorectal mucosa (N1-N4) and carcinoma (T1-T4). Immunoblots were used to detect CTSB expression in late stage colorectal cancer tissues. CTSB expression in advanced stages was significantly higher compared to that in early stages of colorectal cancer (data not shown). β-actin was used as a loading control.

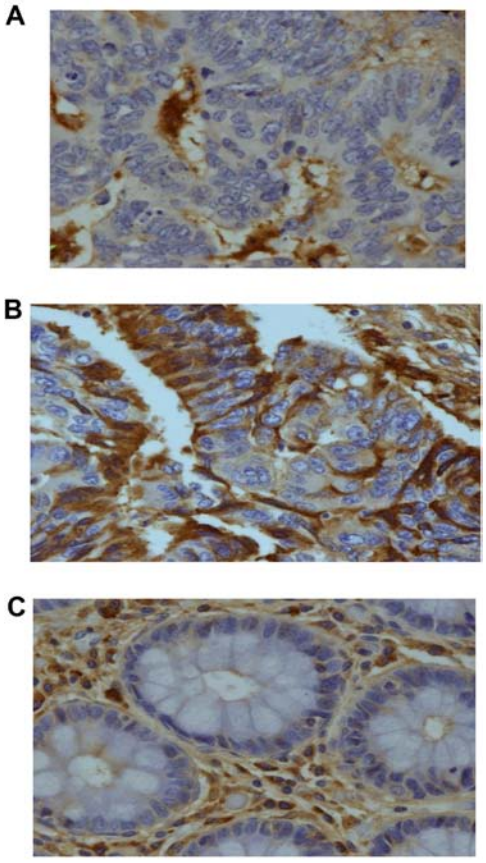

Figure 4. Immunohistochemical staining for cathepsin B. (A) A group of colonic adenocarcinoma cells showing positive membranous and cytoplasmic staining for CTSB in a late stage patient. (B) A group of colonic adenocarcinoma cells showing negative staining for CTSB in early stage of colorectal cancer. Note the presence of focal positive non-significant staining in some inflammatory cells. (C) Photomicrograph showing normal non-neoplastic large bowel mucosa with negative staining for CTSB. Note the positive background staining in some plasma cells. IHC staining, magnification, x600.

adjacent normal colorectal tissue (P<0.05). CTSB gene expression was also elevated in late stage tumor samples, including patients with lymph node metastases, when compared to early stage (P=0.0006). The mRNA level in early stage CRC was significantly lower (0.04±0.01) than the mRNA level in late stage tissues (0.07±0.02) (Fig. 2). These findings suggest that CTSB mRNA was significantly higher in late stage CRC similar to the differences noted in the serum CTSB levels.

Cathepsin B protein expression in tumor tissues. CTSB protein levels were analyzed in early vs. late stage CRC tumor samples, and vs. adjacent normal tissue. Intracellular CTSB protein expression was increased in late stage when compared to adjacent normal mucosa. No differences were noted in early cancer stages vs. adjacent normal tissue. The intracellular/active form of CTSB was highly expressed in late stage CRC samples (Fig. 3). These findings suggest that CTSB is significantly overexpressed in the late stage of CRC. Therefore active CTSB expression during late disease stage (stage III and IV) may have value as a biomarker for late stage CRC including patients with lymph node metastases.

Immunostaining analysis of cathepsin B. We performed immunohistochemistry of CTSB on tissue microarray preparations of our paired tumor and adjacent normal specimens. Positive cells displayed red-brown staining in the cytoplasm. Late stage tumor tissues contained many CTSB-positive tumor cells (Fig. 4C). In contrast only a few were noted in early CRC stage samples (Fig. 4B). Normal colorectal tissues contained very few positive epithelial cells or macrophages (Fig. 4C). We performed a semi-quantitative analysis on patient samples, and 80% of late stage CRC samples showed strong positive staining while only 5% samples exhibited strong staining in early stages (P<0.02). The few early stage samples with significant staining may be in transition from early to late stage. These results demonstrate that CTSB expression is significantly increased at both the transcription and translation levels likely giving rise to increased serum CTSB levels observed in late stage CRC.

Discussion

To date, tumor node metastases (TNM) staging is considered to be the 'gold standard' for tumor classification. It offers valuable prognostic information and a guide to necessary therapeutic decisions. However, patients presenting at a late cancer stage are also a heterogeneous group which makes the

relationship between the TNM stage and patient prognosis very complex (17). Stage II patients who do not receive postoperative adjuvant chemotherapy sometimes show similar disease outcome as stage III patients. Taking these considerations into account, other biomarkers may be useful as an adjunct to the TNM classification for the prediction of responses to therapy and for the planning of personalized and 'tailor made' care (6).

In the present study, we investigated the level of CTSB in blood and tissues using different quantitative immunoassays to evaluate the possible use of CTSB as a biomarker in a specific ethnic group. The present study showed that higher serum levels of CTSB were associated with late stage colorectal cancer and lymph node metastases which is in agreement with the study of Kos *et al* who reported a significant correlation between serum CTSB and late stage colorectal cancer (CRC) (27).

In addition, at the mRNA level, significant differences in CTSB values were noted in late stage cancer when compared to early stage and healthy control. This is in contrast to the results published by other groups (28,29) in which high tumor tissue mRNA was found to be increased in early stage CRC (28,29). To focus on actual protein expression, we investigated CTSB by western blotting in order to confirm that intracellular/active CTSB was highly expressed in late stage disease when compared to early stage tumors and to adjacent normal mucosa. Recently, Bian *et al* found increased CTSB mRNA and protein levels in CRC tumors irrespective of tumor stage (24). These data are reminiscent of the immunohistochemistry data reported by Chan *et al* which showed increased CTSB protein expression in the majority of CRC analyzed patients (558 cohort) and that this was also independent of the tumor stage (25).

To date, numerous published analyses conclude that CTSB plays a key role in the invasiveness of various carcinomas (9,10,15,31). Numerous studies have also reported CTSB expression in CRC (15-17,25,27,32-34). Cathepsin B has been reported to be an accurate indicator and a marker to predict aggressiveness of many tumors such as advanced endometrial cancer, glioblastoma cell lines, breast and advanced endometrial cancer, and pancreatic adenocarcinoma (37-40).

Various studies concluded that an increased CTSB level is associated with earlier local invasion than with the later colonization of the metastatic process (30,31). However, other published studies have reported seemingly contradictory results, that is increased CTSB was correlated with colonizing potential and tumor cell metastases (35). Specifically, Campo *et al* reported that increased expression of CTSB correlates with tumor progression and reduced survival of colorectal patients (36). These discrepancies in the results of different investigations could be due to variations in the genetic background of the populations studied.

In summary, our data provide support for clinical application of preoperative serum CTSB levels in the detection of late stage CRC. Serum CTSB concentrations were significantly higher in patients with advanced CRC and local metastases. Although future comprehensive studies on larger cohort numbers are required for a definitive conclusion, the preoperative serum CTSB level may be useful as a diagnostic and/or predictive biomarker for lymph node metastases in patients with CRC at least in the Saudi population. It is reasonable to anticipate that CTSB detection in advanced stages may have therapeutic benefits as well in CRC. High expression of CTBS in the tumor microenvironment particularly in advanced stages can offer significant therapeutic benefit when using CTBS inhibitors in the treatment of CRC. CTSB inhibitors could be used in combination with known drugs or with nanoparticles as nanotechnology significantly improves drug bioavailability and the therapeutic index in cancer therapy. Combined therapy may improve the therapeutic efficacy of the drug and reduce off-target effects in order to enhance patient outcome.

To the best of our knowledge, the results of our pilot study for the first time demonstrate the clinical utility of CTSB as a blood and tissue-based biomarker to assess the stages in CRC patients from Saudi Arabia. Thus, investigating CTSB as a biomarker in a multi-central larger cohert may be useful in monitoring the course of disease severity and treatment and, therefore contribute to better management of CRC.

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