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RESEARCH ARTICLE

Peripheral blood guanylyl cyclase c (GCC) expressions are associated with prognostic parameters and response to therapy in colorectal cancer patients

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Abstract Guanylyl cyclase C (GCC) is expressed exclusively in normal intestinal mucosal cells, primary and metastatic colorectal cancers (CRC). The aim of this study was to determine the possible association between the GCC expressions in peripheral blood, prognostic parameters and response to chemotherapy in CRC patients. Fourty-nine metastatic CRC patients and 41 healthy controls with similar age and sex were included to this study. Peripheral blood GCC expressions are measured by the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Interstingly, no GCC expression was measured in healthy controls but GCC expressions of the patients were detectable. Although there was a significant reduction in GCC expressions in 30 patients with regression (from 5.46±4.12 to 0.06±0.03, $p<0.0001$), marked increase in GCC expressions was observed in 19 patients with progression following chemotherapy (from 0.43±0.19 to 1.38±0.52, $p=0.0174$). Significant correlation was found between the GCC expressions and carbohydrate antigen 19–9 (CA19-9) levels ($p=0.0041$) in 30 patients with regression before chemotherapy. Marked correlation was also detected between the GCC expressions and carcinoembryonic antigen (CEA) levels ($p=0.0072$) in 19 patients with progression before chemotherapy. The results of the present study suggest that peripheral blood GCC expressions along with CEA and CA19-9 can be used to determine the early respose to chemotherapy in patients with metastatic CRC. These findings imply that higher expression of GCC in peripheral blood seems to be an indicator of good therapeutic response to chemotherapy and remission. Monitoring the peripheral blood GCC expressions may allow employing different treatment options to metastatic CRC patients.

Keywords Guanylyl cyclase C · Progression · Regression · Metastasis · Response to chemotherapy

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Introduction

Colorectal cancer (CRC) is an important global health problem. An important variability in survival between countries has been observed. Differences in survival were explained to a large extent by differences in stage at diagnosis [1]. CRC is the third most common cancer and the fourth most frequent cause of cancer death worldwide [2]. Cancer is the second cause of death in Turkey, and CRC incidence is approximately seven per 100,000, with approximately 5,000 new cases and 3,200 deaths annually [3]. Patients with CRC fall broadly into two groups at time of presentation. The first group has either nonresectable or disseminated disease; the other two-thirds of patients will undergo resection.

It has now been well established that CRC arises as a consequence of the progressive accumulation of genetic

and epigenetic alterations that drive the transformation and progression of normal colonic epithelial cells to cancer. Despite the effective combinations of chemotherapy in the first-line treatment with a response rate of 40%, the majority of patients will display varying levels of resistance and will not benefit from the treatment. The need for individualized treatment is accentuated by the development of an increasing number of new anticancer agents. In consequence, there will be a substantial need for reliable predictive markers of response to the effect of regimens in order to improve the clinical outcome, rational drug use, and avoid unnecessary toxicity [4]. Serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19-9) are commonly used as classical tumor markers in CRC patients. Serum CEA is the marker of choice for monitoring the response of metastatic disease to systemic therapy, while preoperative CA19-9 is useful in predicting patient prognosis in stage IV CRC patients [5].

Guanylyl cyclase C (GCC) is the receptor for heat-stable enterotoxins and guanylin peptides. Guanylin, uroguanylin and lymphoguanylin are three closely related guanylin peptides discovered initially in the gastrointestinal tract that regulate electrolyte and water transport in intestinal and renal epithelia through a cyclic GMP-dependent mechanism, and they also have an important role in the control of intestinal epithelial cell proliferation and differentiation [6–9]. Guanylyl cyclase activity of the receptor GCC is modulated by adenosine triphosphate (ATP), and exposure of GCC-expressing cells to GCC agonists (uroguanylin analogs) results in cell cycle arrest and/or apoptosis [10].

GCC is expressed by intestinal and CRC cells but not by extraintestinal tissues or tumors [11]. GCC mRNA expression has been identified in every primary and metastatic colorectal tumor obtained from patients regardless of tumor grade, stage or anatomic location [12–14]. Although involvement of certain signal transduction pathways in CRC has been suggested [15], there are only limited studies investigating the role of peripheral blood GCC expression in CRC patients [16, 17]. Moreover, no study established the peripheral blood GCC expression changes in response to chemotherapy. Therefore, the purpose of the present study was to investigate a possible association between GCC expression and CRC in a Turkish population.

Methods

Study populations

Fourty-nine metastatic stage IV CRC patients (15 female and 34 male, age: 53.65±12.17 years, 27 rectum and 22 colon carcinoma) and 41 healthy controls (18 female and 23 male, age: 52.27±10.63 years) were included in the study at the Department of Oncology, University Hospital in Gaziantep. The study was approved by the local ethics committee, and written informed consent was obtained from all patients before entering the study. Eligibility criteria were histologically confirmed metastatic CRC (adenocarcinomas) and indication for first-line treatment with chemotherapy, Eastern Cooperative Oncology Group performance status of two or less, adequate organ function, age >18 years and life expectancy of 1 year or more. Chemotherapy regimens including oxaliplatin-based therapy, capecitabine, irinotecan-based therapy, and also concomitant chemoradiotherapy were given until disease progression, unacceptable toxicity, or a patient's refusal to continue treatment. Patients received folinic acid, fluorouracil, oxaliplatin (FOLFOX; 22.4%); FOLFOX plus bevacizumab (8.2%); folinic acid, fluorouracil, and irinotecan (FOLFIRI; 4.1%); FOLFIRI plus bevacizumab (8.2%); irinotecan (2%); mitomycin C, uracil/tegafur (UFT) plus folinic acid (4.1%); capecitabine plus oxaliplatin (XELOX, 10.2%); XELOX plus bevacizumab (2%); UFT plus bevacizumab (2%); capecitabine (2%); and folinic acid, fluorouracil plus concomitant chemoradiotherapy (34.8%). Tumor staging was recorded according to the tumor–node–metastasis (TNM) staging system.

Blood samples

From all patients, 5 ml of peripheral blood sample was collected by venapuncture into sterile siliconized vacutainer tubes with 2 mg/ml disodium ethylenediaminetetraacetic acid. All samples were stored at −20°C until use.

RNA isolation and gene expression

To confirm the presence and expression of GCC in the blood, we extracted mRNA from blood samples. RNA was extracted from leukocytes using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer (https://www.roche-applied-science.com/pack-insert/1828665a.pdf). cDNA was produced with the First Strand cDNA Synthesis Kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's protocol. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed by the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany) with GCC primers (Table 1) and HPRT1 (housekeeping gene) (TaqMan MGB probes, FAM dye-labeled) and TaqMan Universal PCR Master Mix. All primers (Table 1) were designed by using an online program (www.roche-applied-science.com/sis/rtpcr/upl). These sequences were compared with the sequences at NCBI database and validated. GCC primers were synthesized at IDT (Integrated DNA Technologies, Belgium), and

Table 1 Housekeeping gene and primer sequences and probes for GCC genes

Gene	Primer sequence	UPL probe	NCBI nucleotide number
HPRT1	5′-tgaccttgatttattttgcatacc-3′ 5′-cgagcaagacgttcagtcct-3′	Probe 23	NM_000194.1
GGC	5′-gcattggagttcactctggtc-3′ 5′-aaatagacaataacgaggcatcttg-3′	Probe 67	NM_004963.2

Universal Probe Library (UPL) probes (Roche Diagnostics, Mannheim, Germany) were used in this study. The cycling program was carried out after a denaturation step at 95°C for 10 min through 45 cycles (denaturation at 95°C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 1 s), and 1 cycle at 40°C for 30 s, with a maximum ramp rate of 20°C/s. All samples were prepared twice and each preparation set up in triplicate. Data were analyzed using the $2^{-\Delta Ct}$ method, according to the formula: $\Delta Ct = Ct_{GCC} - Ct_{HPRT1}$, where Ct= threshold cycle.

CEA and CA19-9 levels

CEA and CA19-9 was measured by chemiluminescence methods with Immulite 2000 Immunoassay System (Siemens Healthcare Diagnostics, Germany) from peripheral blood samples.

Statistical analyses

Results are expressed as mean±standard error of mean (SEM). All statistics were calculated using the Graph Pad Instat statistical software (version 3.05, GraphPad Software Inc., San Diego, CA, USA). Mann–Whitney *U* test was used for comparisons of the differences between values of two groups. Wilcoxon matched pair signed ranks test was used for comparisons of the values measured before and after chemotherapy. A Fisher's exact test was used to detect significant differences in the rate of expression. Pearson test was used to identify the correlations. All *P* values referred to two-tailed tests and were considered significant when $P<0.05$.

Results

Expression of GCC in the peripheral blood of metastatic CRC patents at mRNA levels (3.508±17.702, $n=49$) was detected. However, no expression was found in the peripheral blood of the control group. GCC mRNA was detected in 21/30 (70%) patients with regression. In patients with progression, GCC mRNA was expressed in 7/19 patients before chemotherapy, but this rate increased to 17/19 after chemotherapy. GCC expression could be detected in nonresponders with a rate of 36.8% and 89.5% before and after the chemotherapy, respectively. *P* value for this comparison is 0.0019 with Fisher's exact test. Peripheral blood GCC expression was high in patients responding to chemotherapy (5.46±22.54, $n=30$), but its level was markedly low in patients resistant to chemotherapy (0.43±0.84, $n=19$, $P=0.038$). Peripheral blood mRNA GCC content was markedly reduced with the third chemoterapy administration in 30 patients with regression (Fig. 1). On the other hand, peripheral blood

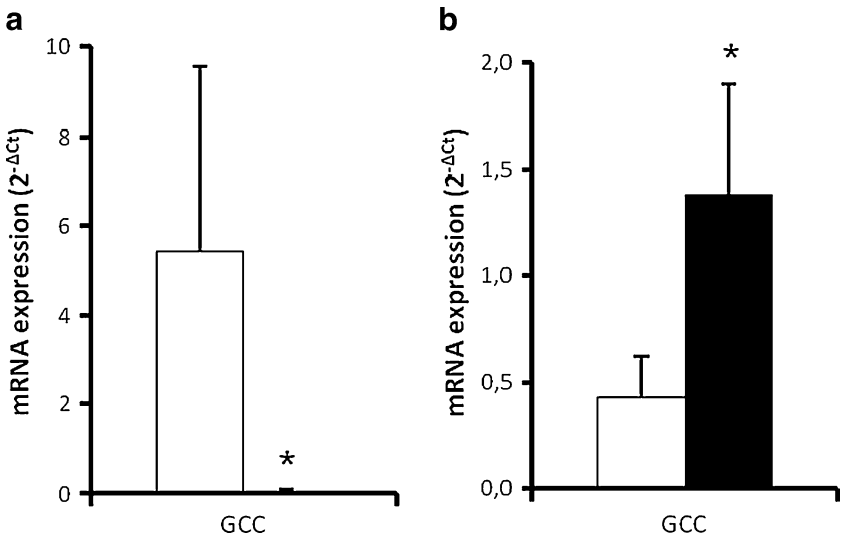

Fig. 1 Comparions of the peripheral blood mRNA GCC expressions before (*open bars*) and after (*solid bars*) chemoterapy in 30 patients with regression (**a**), and in 19 patients with progression (**b**). After chemotherapy indicates the values after the third chemotherapy cycle. *$P<0.0001$ and $P=0.0174$ are for **a** and **b**, respectively

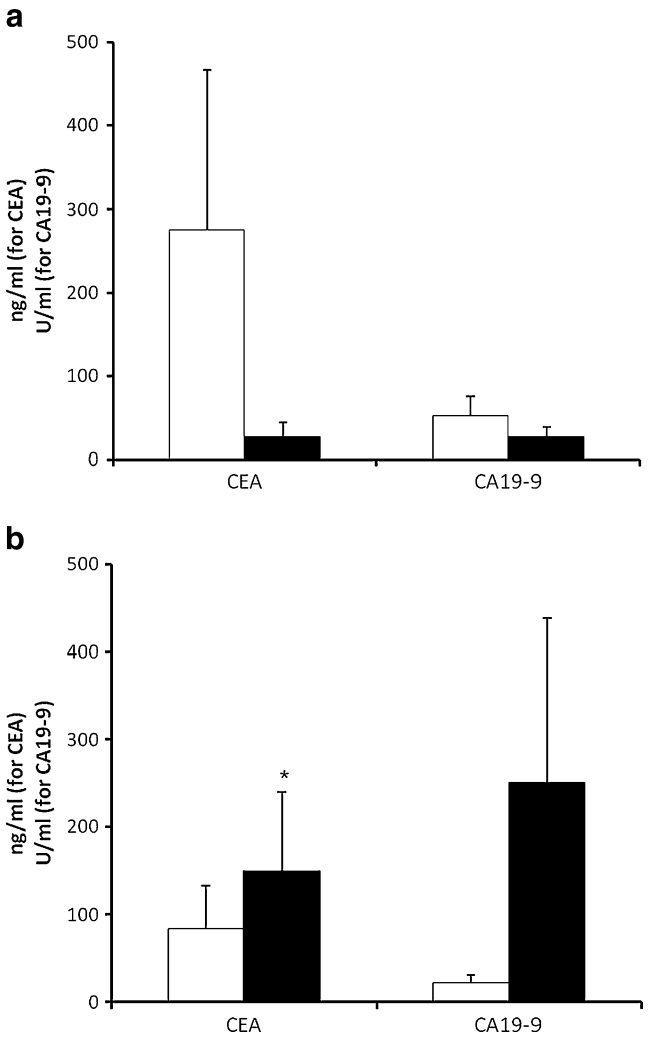

Fig. 2 Comparions of the peripheral blood CEA and CA19-9 levels before (*open bars*) and after (*solid bars*) chemoterapy in 30 patients with regression (**a**), and in 19 patients with progression (**b**). After chemotherapy indicates the values after the third chemotherapy cycle. **P*=0.0090

mRNA GCC content was markedly augmented with the third chemoterapy administration in 19 patients with progression (Fig. 1). Although CEA and CA19-9 levels appeared to decrease after chemoterapy, these levels were not significantly changed (Fig. 2). CEA levels were also significantly increased following chemotherapy in these patients. However, elevated CA19-9 values did not reach statistically significant levels in patients with progression (Fig. 2).

GCC mRNA expression changes were independent of chemotherapy and/or radiotherapy regimens. Significant correlation between GCC expressions and CA19-9 levels was detected before chemoterapy (Table 2). This correlation was also present in patients with regression (Table 3), but not in patients with progression (Table 4). Only marked correlation was found between GCC mRNA content and CEA levels before chemotherapy in patients with progression (Table 4).

Table 2 Correlations between the peripheral blood GCC expression, CEA and CA19-9 levels before and after chemoterapy in all patients

Groups	Correlation coefficient (r)	Coefficient of determination (r^2)	P value
Before chemotherapy			
GCC↔CEA	−0.0099	$9.987\ 10^{-5}$	0.9457
GCC↔CA19-9	0.5025	0.2525	0.0002
After the third chemotherapy cycle			
GCC↔CEA	0.1601	0.0256	0.2718
GCC↔CA19-9	−0.0639	0.0041	0.6625

Table 3 Correlations between the peripheral blood GCC expression, CEA and CA19-9 levels before and after chemoterapy in 30 patients with regression

Groups	Correlation coefficient (r)	Coefficient of determination (r^2)	P value
Before chemotherapy			
GCC↔CEA	−0.0294	0.0008	0.8776
GCC↔CA19-9	0.5083	0.2584	0.0041
After the third chemotherapy cycle			
GCC↔CEA	−0.0603	0.0036	0.7514
GCC↔CA19-9	0.3332	0.1110	0.0720

Discussion

Our study is the first in literature to investigate the gene expression levels of GCC in peripheral blood of metastatic CRC patients in response to chemotherapy. Our data have shown that GCC was expressed in peripheral blood of metastatic CRC patients, but not in healthy controls. Peripheral blood mRNA GCC content was markedly

Table 4 Correlations between the peripheral blood GCC expression, CEA and CA19-9 levels before and after chemoterapy in 19 patients with progression

Groups	Correlation coefficient (r)	Coefficient of determination (r^2)	P value
Before chemotherapy			
GCC↔CEA	0.5951	0.3542	0.0072
GCC↔CA19-9	−0.2019	0.0408	0.4072
After the third chemotherapy cycle			
GCC↔CEA	0.0754	0.0057	0.7591
GCC↔CA19-9	−0.1788	0.0320	0.4639

reduced with chemoterapy in patients with regression, but increased in patients with progression.

GCC, a member of the guanylyl cyclase family of receptors, is specifically expressed only in intestinal mucosal cells [18, 19]. The GCC is constitutively expressed in the apical membranes of the intestine and its expression is universally preserved in primary colorectal tumors and their metastases [12, 13, 20–22]. Examination of more than 300 surgical specimens demonstrated that GCC was expressed by all primary and metastatic colorectal cancer cells but not by any other extraintestinal tissues or tumors [12, 13, 20, 21]. Previous studies has demonstrated expression of GCC mRNA in lymph nodes, and suggested that analysis of GCC mRNA expression may enhance the accuracy of colorectal cancer staging [11–13, 20, 23]. These studies showed that lymph node expression of GCC mRNA, a biomarker for colorectal cancer cells in extra-intestinal tissues, is associated with disease recurrence in patients with histologically negative nodes (stage II disease).

Predictive markers will aid the identification of patients who are likely to benefit from a specific treatment, and have the potential to guide the clinician in prescribing the chemotherapy most likely to provide benefit. Predictive markers may play a significant role in the first-line treatment of metastatic disease and also in the neoadjuvant setting, allowing the selection of chemotherapy with a high probability of inducing a significant response. Cytotoxic chemotherapy remains the most effective treatment for inoperable metastatic CRC. Currently, there are no recommended molecular markers for predicting the efficacy of standard of care chemotherapy in metastatic CRC [4]. We observed high peripheral blood GCC expression levels in regression and low levels in progression. Therefore, our results may suggest that peripheral blood mRNA GCC content may be a predictive marker for metastatic CRC. Appropriate chemotherapy in the setting of distant metastasis will also prolong survival and improve quality of life.

Prognostic factors are associated with overall disease outcome and are largely independent of treatment. Several prognostic markers are in routine clinical use (CEA, T and N stage, histopathology). Our data imply that peripheral blood GCC expression has a potential to be a prognostic factor in metastatic CRC, since peripheral blood mRNA GCC content changes with chemoterapy during regression or progression of the carcinoma.

In conclusion, the findings of the present study suggest that peripheral blood GCC expression along with CEA and CA19-9 levels can be used to determine the early respose to chemotherapy in patients with metastatic CRC. Our data showed that baseline GCC mRNA levels are significantly higher in responders compared to the resistant patients. After the chemotherapy, GCC mRNA levels seem to decrease in responders and increase in nonresponders. Our results also suggest that GCC may serve as a prognostic and predictive marker, and peripheral blood mRNA GCC content may be used to monitor the response to chemotherapy. Our sample was relatively small and limited the generalizability of our observations. Future studies of larger patient samples are needed to establish the importance of peripheral blood guanylyl cyclase C expression for detecting colorectal cancer micrometastases and predicting the response to chemotherapy. However, our data may suggest that peripheral blood mRNA GCC content determination could enter clinical practice in metastatic CRC treatment.

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