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Research Article

Clinical Significance of NGAL, MMP-9, and VEGF in Colorectal Cancer Patients

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ABSTRACT

Objectives. We aimed to investigate the levels of neutrophil gelatinase associated lipocalin (NGAL), matrix metalloproteinase (MMP-9) and vascular endothelial growth factor (VEGF) among colorectal cancer (CRC) patients especially among pre and post-treated individuals. Materials and methods. A total of 162 CRC patients were enrolled and they were divided according to their clinical stage into early CRC stage (stage I, II, and III, n=90) and advanced stage (stage IV, n=72). A group of healthy individuals (n=57) were also included as controls. Blood samples were collected from them before and after they received treatments as well as from the control group where NGAL, MMP-9, VEGF, CEA and CA19-9 were asses in their sera using Enzyme linked immunosorbent assay. Results. Serum levels of NGAL, MMP-9, their ratio (NGAL/MMP-9) and VEGF were significantly higher in CRC patients as compared with control individuals. By using the receiver operating characteristic (ROC) curve for detection of the diagnostic efficacy, NGAL and MMP-9 were superior to other markers and routine tumor markers (CEA and CA19-9) in detection of CRC patients. Levels of investigated biomarkers in both early and advanced post-treated CRC were significantly decreased than in pretreated patients as compared to CEA and CA19-9 as they did not report significance decrease. Conclusion. Our findings suggest the usefulness of serum NGAL, MMP-9, VEGF in diagnosis of CRC patients and their superiority over CEA and CA19-9 as adjuvant biomarker for treated patients.

Keywords: NGAL, MMP-9, VEGF, Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common gastrointestinal cancers worldwide[1], globally the CRC frequency varies as the highest incidence rates are in Australia and New Zealand, Europe and North America, and the lowest rates are found in Africa and South-Central Asia[2]. These geographic differences appear to be attributable to differences in dietary and environmental exposures that are imposed upon a background of genetically determined susceptibility. Despite extensive research in this field, the mechanisms underlying colorectal cancer progression and metastasis are still unknown. If detected at an earlier stage, the majority of CRC patients can be cured successfully; because there is a strong correlation between the tumor stage of diagnosis and the 5-year survival rate[3]. With the developments in modern surgery and chemical therapeutics, including molecular-targeted therapy, the 5-year survival rate is still not high, especially for stage IV colorectal cancer patients[4]. The early diagnosis of CRC is always difficult because of the late onset of symptoms; therefore, many screening tests have been developed, such as fecal occult blood test and colonoscopy. TNM staging is still the traditional technique for prognosis predication in the majority of cases. Therefore, it is important to find an effective tumor biomarker, which can be useful for both diagnosis and prognosis of colorectal cancer. Neutrophil gelatinase associated lipocalin (NGAL), also referred to as lipocalin 2, is a 25-kDa secretory glycoprotein belongs to a large family of lipocalins, which are a group of small extracellular proteins and characteristically bind small lipophilic molecules because of their common three-dimensional β-barrel structure and have great functional diversity[1]. It has been reported that NGAL mRNA and protein expression are both reported to be elevated in tumor tissue when compared to adjacent normal tissue appear in mucosa and its expression is localized to the tumor margins[5]. Although more recent studies have confirmed this pattern of NGAL expression in human CRC tissue, its role in colorectal tumorigenesis is still controversial[6,7]. Proteolytic degradation of extracellular matrix (ECM) is observed in many physiological conditions, it plays a role in development of malignant tumors as a key step in the regulation of cancer proliferation[8]. It was shown that tumor cells, including CRC, are able to produce and release matrix metalloproteinase 9 (MMP-9), a proteolytic enzyme capable of degrading the basement membrane and type IV collagen in the ECM. Degradation of collagen by MMP-9 is involved both in tumor invasion and metastasis[9]. Increased expression of this enzyme in CRC cells was associated with enhanced invasiveness of tumor[10].

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Previously it was reported that NGAL was originally identified as a glycoprotein in complex with matrix metalloproteinase-9 (MMP-9) in human neutrophils. It bounds with MMP-9 covalently to form a 135-kDa disulfide-linked heterodimer. NGAL was shown to be able to bind and stabilize MMP-9 and facilitate intracellular iron delivery, thus playing a regulatory role in iron-dependent gene transcription in kidney development[11]. It was reported that plasma NGAL and NGAL/MMP-9 complex may act as diagnostic adjuvant biomarkers for pelvic inflammatory disease[12]. To date, few data are available on the significance of the ratio between NGAL/MMP-9 in CRC patients. Angiogenesis has been known to play an important role in the development of tumor growth and lymph node metastasis. Increased angiogenesis in the primary tumor of CRC has been associated with poor prognosis and relapse of disease[13]. Vascular endothelial growth factor (VEGF) family is the most widely investigated and most specific regulator of angiogenesis, which consist of six members. They increase vascular permeability and promote the formation of new blood vessels in tumors and thus are regarded as the main growth stimulatory factors in the tumor-related angiogenesis[14]. Authors aimed in this preliminary study to investigate the clinical implication of NGAL, MMP-9, their ratio and VEGF in CRC patients. Also to compare the diagnostic efficacy of the above mentioned circulating markers with classical tumor markers (carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) using the receiver operating characteristic (ROC) curve. Moreover, authors designed to study the relation between these circulating markers in early and late stage CRC before and after they received treatment to assess their future usefulness as follow-up markers.

MATERIALS AND METHODS

Enrolled individuals and sample collection

The study has been approved by the local Ethics Committees. Informed consent of the individuals had been obtained prior to their inclusion in the study. A total number of 219 Individuals were enrolled in the current study. They were divided into 162 patients with colorectal cancer (CRC) and 57 healthy individuals as controls. The CRC group comprised of (n= 81) females and (n=81) males, all were of matched ages (F= 2.268, *P*=0.106). The diagnosis of CRC was confirmed by microscopic examination of the tumor samples obtained during colonoscopy, biopsy, and/or surgery. The subjects who had suffered a heart attack or heart failure were not included in the study. Moreover, the patients with extra-intestinal tumors and those after preoperative radio-chemotherapy were excluded from the study. Clinical staging of CRC was determined based on the UICC-TNM classification (Sobin and Wittekind 2002). Stages I, II and III were collectively termed the early stage CRC [E-CRC] group (n = 90) and stage IV as advanced CRC [A-CRC] group (n = 72). Blood samples were collected from CRC patients before they have received any type of treatments, then 3 days after they have been treated. A group of matched age healthy individuals were enrolled in the study, they were (n=28) females and (n=29) males. Their clinical diagnosis were based on Physical examination, blood tests, chest x-rays, abdominal ultrasound, and computed tomography. Blood samples were collected from enrolled individuals and left to stand at room temperature for 30 min prior to centrifugation (1.200 g, 10 min, at room temperature). Then serum fractions were then aliquotted and stored at -80°C until required.

Biochemical analysis

The NGAL, MMP-9 and VEGF levels in the serum samples were analyzed by human NGAL, MMP-9 and VEGF ELISA kits (R&D Systems, Abingdon, UK), respectively. From each sample, 100 µl was directly transferred to the micro-test strip wells of the ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm using GloMax®-Multi-Detection System (Promega Corporation, Madison, USA). Serum concentrations of CEA and carbohydrate antigen 19-9 (CA 19-9) were measured by enzyme immunoassay kits (Abbott, Chicago, Illinois).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) (SPSS, version 10.0 Chicago, IL). The differences between control and CRC groups were determined by the non-parametric Mann-Whitney U test and among the entire groups using Kurskal- Wallis H test. One-way ANOVA was used to determine statistical significance between levels of the investigated biomarkers and patient characteristics. Receiver operating characteristic (ROC) curve (15) was performed to assess the clinical performance of the investigated biomarkers as compared to the routine markers as diagnostic marker.

RESULTS

The enrolled individuals were comprised of 162 CRC patients and 57 controls of matched ages and a similar ratio of male to female individuals. The CRC group was divided according to their clinical staging into early stage (n= 90) and advanced stage (n=72), according to the gender status; both groups were comparable (male/female; E-CRC: 39/51, A-CRC: 42/30). Those with E-CRC (median 45 years, range: 21-61 years) were reported to be of older ages than A-CRC (median 38 years, range: 21-65 years) at a significant level (F=4.5, *P*=0.035). Increased serum levels of the investigated circulating biomarkers were observed in CRC patients (both with E-CERC or A-CRC) as compared to healthy individuals as shown in Table (1). When authors compared the levels of the investigated biomarkers among the entire CRC groups, all reported a significant increased in E-CRC as compared to A-CRC Significant apart from NGAL/MMP-9 which did not did not reach a significant value (Table 1). The diagnostic efficacy for the investigated circulating biomarkers (NGAL, MMP-9, NGAL/MMP-9 and VEGF) were compared with the routine tumor markers (CEA, and CA19-9) using the receiver operating characteristic (ROC) curve (Figure 1). Based on this analysis, the highest AUC was detected for MMP-9 as well as the highest sensitivity

Table 1: Median levels of circulating biomarkers for both control and colorectal patients based on their clinical stage.

Circulating biomarkers	Control n=57	E-CRC n=90	A-CRC n=72	C/LCRC	C/ACRC	LCRC/ACRC
NGAL (ng/ml)	78	202.5	248.5	F=35, *P*<0.001	F=34, *P*<0.001	F=15.8, *P*<0.001
MMP-9 (ng/ml)	329	777	900	F=143, *P*<0.001	F=50, *P*<0.001	F=26, *P*<0.001
NGAL/MMP-9 (ng/ml)	0.22	0.28	0.22	F=7.48, *P*=0.007	F=7.47, *P*=0.007	F=1.38, *P*=0.241
VEGF (ng/ml)	4.1	6.3	10.5	F=39, *P*<0.001	F=35, *P*<0.001	F=38, *P*<0.001
CEA (ng/ml)	4	4	23	F=9.9, *P*=0.002	F=346, *P*<0.001	F=500, *P*<0.001
CA19.9 (ng/ml)	7	14	18	F=62, *P*<0.001	F=97, *P*<0.001	F=7.6, *P*=0.007

C, control; E-CRC, localized colorectal cancer; A-CRR, advanced colorectal cancer.

Table 2: Sensitivity, specificity and AUC for investigated biomarkers using ROC curve analysis.

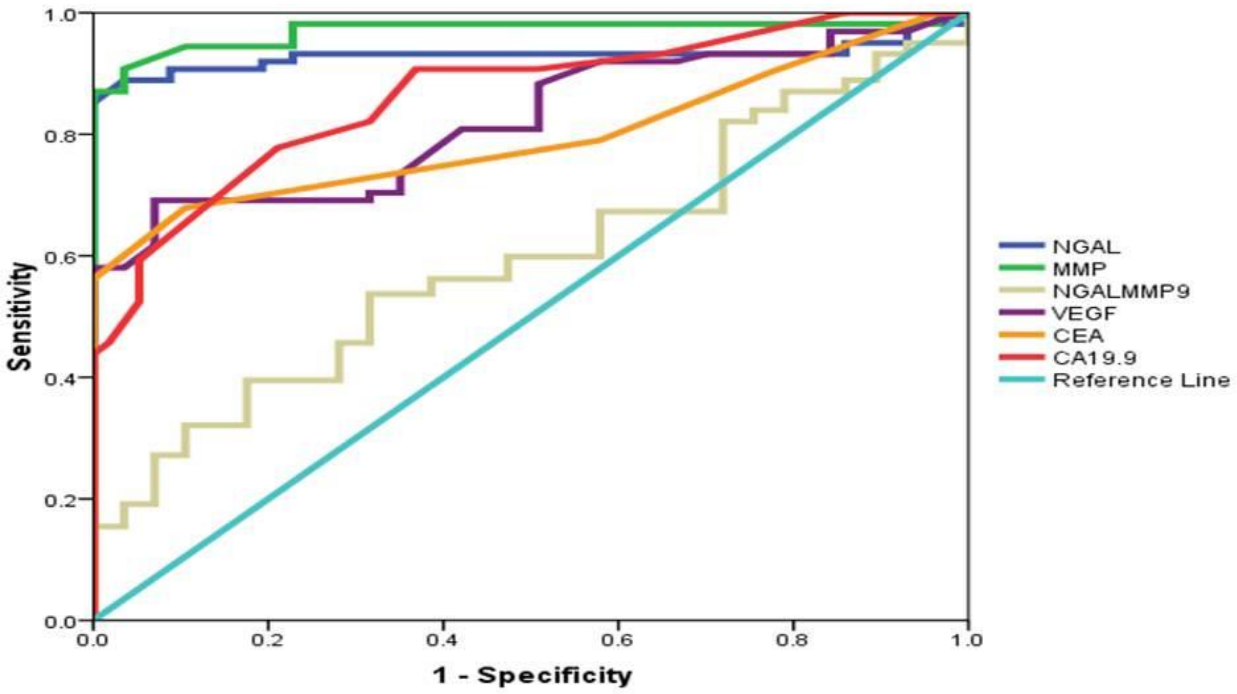
	Sensitivity	Specificity	AUC	SE	% CI	*P*-value
NGAL (ng/ml)	96.5	89.5	0.929	0.019	0.893 – 0.966	0.0001
MMP-9 (ng/ml)	96.5	92	0.97	0.011	0.948 – 0.992	0.0001
NGAL/MMP-9 (ng/ml)	46.9	70	0.596	0.04	0.518 – 0.675	0.031
VEGF (ng/ml)	69.1	93.3	0.822	0.027	0.769 – 0.876	0.0001
CEA (ng/ml)	56.2	99	0.795	0.029	0.738 – 0.852	0.0001
CA19.9 (ng/ml)	52.7	76.7	0.863	0.025	0.814 – 0.912	0.0001

Table 3: Mean ranks of circulating biomarkers for both localized and advanced colorectal patients before and after treatments.

Circulating biomarkers	E-CRC (n=90)			A-CRC(n=72)		
	Pretreated	Post-treated	Statistics	Pretreated	Post-treated	Statistics
NGAL (ng/ml)	64.4	26.6	*P*<0.001	53.1	19.8	*P*<0.001
MMP-9 (ng/ml)	55.6	35.4	*P*<0.001	42.7	30.3	0.012
NGAL/MMP-9 (ng/ml)	57	34	*P*<0.001	50.3	22.7	*P*<0.001
VEGF (ng/ml)	53.8	37.2	0.003	46.4	26.6	*P*<0.001
CEA (ng/ml)	46.8	44	0.64	38.5	34.5	0.415
CA19.9 (ng/ml)	47.5	43.5	0.46	38.3	34.6	0.455

C, control; E-CRC, localized colorectal cancer; A-CRR, advanced colorectal cancer.

and specificity followed by NGAL then VEGF as compared to other routinely used markers (Table 2). The correlation between the levels of the investigated markers (NGAL, MMP-9, their ratio and VEGF) and routine tumor markers in CRC patients revealed that NGAL, MMP-9 and VEGF were significantly correlated with CEA (R=0.253, 0.307, 0.305, respectively at *P*<0,.) apart from NGAL/MMP-9 (R=0.091, at *P* = 0.249). Regarding their correlation with CA19.9; only VEGF and NGAL/MMP-9 reported significant difference (R=0.229, 0.166, respectively at *P*<0.03) while NGAL and MMP-9 reported no significant correlation with CA19.9. Interestingly the three investigated markers i.e. NGAL, MMP-9 and VEGF were significantly correlated with each other among the studied population (*P*<0.01). When treatment was considered (Table 3), among those with early CRC patients the levels of NGAL, MMP-9 and their ratio were significantly increased in pretreated patients as compared to their levels in post-treated patients. Although the level of routine tumor markers (CEA and CA19.9) was higher in pretreated as compared to their levels in patients after they received treatment but no significant level was reached. Similarly the levels of the investigated markers (NGAL, MMP-9, NGAL/MMP-9and VEGF) were higher in pre-treated advanced staged CRC patients as compared



Variables	AUC	SE	%CI	*P*-value
NGAL (ng/ml)	0.929	0.019	0.893 – 0.966	0.0001
MMP-9 (ng/ml)	0.97	0.011	0.948 – 0.992	0.0001
NGAL/MMP-9 (ng/ml)	0.596	0.04	0.518 – 0.675	0.031
VEGF (ng/ml)	0.822	0.027	0.769 – 0.876	0.0001
CEA (ng/ml)	0.795	0.029	0.738 – 0.852	0.0001
CA19.9 (ng/ml)	0.863	0.025	0.814 – 0.912	0.0001

Figure 1: Receiver operating characteristic curve for the investigated biomarkers and routine tumor markers.

to their levels in post-treated ones (at $P<0,01$). Although routine tumor
markers were higher in pretreated patents as compared to post-treated advanced CRC patients their levels did not reached a significant value.

DISCUSSION

In this study, serum levels of NGAL, MMP-9, VEGF and routine tumor markers (CEA and CA19-9) were significantly increased in CRC patients as compared to healthy individuals, which is in line with results obtained by others[16,10,14]. To date, few data are available on the significance of the ratio between NGAL/MMP-9 in CRC, thus we analyze the level of their ratio among the studied population. The current results revealed significant elevation of the NGAL/MMP-9 in CRC patients as compared to controls. For detection of diagnostic efficacy of the investigated biomarkers, ROC curve was plotted between control individuals and collective CRC patients (Figure 1), accordingly MMP-9 levels reported the highest AUC followed by NGAL, CA19-9, VEGF then CEA. These findings support the hypothesis that biomarker combination will be preferred than a singly one for better diagnosis of CRC. Moreover, our results reported significant correlation between NGAL, MMP-9 and VEGF among the studied population. The NGAL role in tumorgenesis has been widely investigated however its exact role is unclear. In several experimental studies it has been reported that NGAL aid in regulating cell growth as its expression is highly up-regulated in a variety of proliferative cells such as cancer[17,18]. NGAL was identified as glycoprotein in complex with MMP-9 (NGAL/MMP-9), thus preventing the degradation of MMP-9 and hence increase the MMP-9 activity[11]. Consecutively, MMP activity promotes cancer progression by the degradation of the basement membranes and the extracellular matrix cause the liberation of VEGF, and thus enabling angiogenesis, invasion and metastasis[19]. By investigating our interested circulating biomarkers in early and advanced CRC and compare these results with control individuals, all of them reported significant differences. Based on these findings we may assume that serum concentration of NGAL can reflect the tumor progression and hence can be used as circulating prognostic biomarker for CRC. In a study carried by Kuben and his colleagues[20], they reported that NGAL, MMP-9 and their complexes were abundant in tissue homogenate from primary gastric adenocarcinoma and their levels were significantly

correlated with disease progression suggesting that NGAL has a role in tumor progression through suppression of MMP-9 auto-degradation. Along the same lines, high VEGF levels were significantly elevated in advanced CRC than localized ones, which agreed with previous findings that tumor growth is encouraged by VEGF which binds to endothelial cells and initiates the process of new blood vessel formation[14]. Additionally, authors have investigated the level of circulating NGAL, MMP-9, NGAL/MMP-9 and VEGF in pretreated and post – treated CRC both with early and advanced stage as compared to routine CEA and CA19-9. The significant differences reported between the levels of investigated biomarkers among the CRC patients which as lacking in the levels of CEA and CA19-9 indicates their superiority over the routine tumor marker for following-up CRC patients. In conclusion, the significant elevated levels of investigated circulating biomarkers (NGAL, MMP-9, and VEGF) in CRC as compared to control individuals encourages the use of these biomarkers as supplementary parameters for use alongside CEA and CA19-9 to monitor CRC patients in daily clinical practice. In addition, to the best of our knowledge, this is the study to compare the level of these panels of biomarkers with routinely used tumor markers for post and pre-treated CRC patients which may open a new era for new prognostic markers as well as markers for targeted therapy.

DISCLOSURES

The authors report no conflicts of interest.

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