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Study the Role of Human Neutrophil Gelatinase Associated Lipocalin and Matrix Metalloproteinase-9 in Egyptian Colorectal Cancer Patients

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ABSTRACT

Purposes: Neutrophil gelatinase-associated lipocalin (NGAL), a recently recognized protein that binds to Matrix metalloproteinase-9 (MMP9).There is growing evidence implicating that Neutrophil gelatinase–associated lipocalin and Matrix metalloproteinase-9 play a role in the development and progression of cancers. In this study, we investigated the role of NGAL and MMP-9 in the tumorigenesis and progression of Colorectal cancer (CRC).We showed the correlation between the plasma levels of NGAL and MMP-9 in CRC, and also we studied the effect of treatment with chemotherapy and/or radiotherapy on plasma levels of NGAL and MMP-9 in human CRC. Methods: We used ELISA immunoassay to measure the levels of plasma NGAL and MMP-9 in a cohort consisting of colorectal cancer patients (n=50) and controls (n=30). Cases were assigned into 5 groups: Group 1(Control),Group 2 (Patients with early stages (I,II), who weren't receive chemotherapy and /or radiotherapy), Group 3 (Patients with early stages (I,II), who were received chemotherapy and/ or radiotherapy, Group 4 (Patients with late stages (III, IV), who weren't receive chemotherapy and/or radiotherapy) and Group 5(Patients with late stages (III, IV), who were received chemotherapy and/ or radiotherapy).Results: Both NGAL and MMP-9 plasma levels were highly significantly elevated in all studied groups compared to control group ($P<0.0001$) but no significant differences in plasma levels of NGAL and MMP-9 were found between groups that received treatment with chemotherapy and /or radiotherapy and those not received, or between different stages (Tumor Nodes Metastases staging (TNM)). of CRC .In addition we found significant correlation between levels of NGAL and levels of MMP-9($p< 0.05$). NGAL was positively correlated with MMP-9 (The correlation coefficient between NGAL and MMP-9 was 0.286).Conclusion: Our study suggested that NGAL and MMP-9 may be have screening role but not for prognosis of CRC Patients and they have no value in monitoring the effect of treatment with chemotherapy and/or radiotherapy on CRC patients.

Key words: Human Neutrophil, Lipocalin, Colorectal cancer, Matrix metalloproteinase.

Introduction

Colorectal cancer (CRC) has the fourth most common malignant tumors worldwide (Amin *et al*., 2015). CRC affects men and women of all racial and ethnic groups (Gado *et al*., 2014). In Egypt, colorectal carcinoma is one of the most common malignant neoplasms and the third leading cause of death. It represents about 6% of cancers in Egypt (after bladder, breast carcinoma and lymphoma) (Kamal *et al*., 2012). Currently, due to a frequent lack of early disease specific symptoms and a reluctance to seek medical investigation, many colorectal cancer cases present late when the disease is at a relatively advanced stage. Colorectal cancer often appears to develop slowly over years and, in many cases, there is an initial pre invasive polyp stage. For this reason, unlike some other solid malignancies, colorectal cancer is a good candidate for prevention screening strategies (Coghlin and Murray, 2015). Nearly two-thirds of newly diagnosed cases of CRC have lymph node involvement or metastatic disease (Maier *et al*., 2014); 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 (Wang and Zeng, 2014).

NGAL, also known as lipocalin-2 (LCN2) or 24p3, is a member of lipocalin protein family. NGAL was originally identified as a 25 kDa protein covalently associated with MMP-9 (92 kDa).Formation of this heterodimer enhances and stabilizes the gelatinolytic activity of MMP-9 by preventing MMP-9 from degradation (Weng and Chou, 2015).Its best-known function is as a carrier for a myriad of small hydrophobic ligands .The expression of NGAL is increased in a spectrum of human epithelial cancers, including breast, ovarian, colon, pancreatic, and thyroid cancers. Studies have shown that NGAL can facilitate tumor cell proliferation and invasion (Ding *et al*., 2015). NGAL is also expressed in several adult normal tissues like breast ducts, liver, lungs, trachea, small intestine, bone marrow, thymus, adipose tissue and macrophages. Negligible

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expression of NGAL is observed in the normal pancreas, endometrial glands and peripheral blood leukocytes (Monisha *et al*., 2014).

Matrix metalloproteinase-9 (MMP-9) belongs to a family of zinc-dependent proteases, mostly secreted as inactive pro-enzymes, activated outside the cell upon proteolytic cleavage (lepeta and Kaczmarek, 2015).It was shown that tumor cells, including CRC, are able to produce and release matrix metalloproteinase 9(Mroczko *et al*., 2010). MMP-9 plays a key role in the invasive ability and the degradation of the basement membrane, which is composed of type IV collagen (El Moety *et al*., 2013). Both NGAL and MMP-9 (active and latent) have already emerged as useful biomarkers in a wide array of malignant diseases including breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung and skin cancers (Bouchet and Bauvois ,2014).In this study we investigated the clinical value of NGAL and MMP-9 determination in initiation and progression of CRC and the effect of chemotherapy and/or radiotherapy on plasma levels of NGAL and MMP-9 and this help in determining the role of NGAL and MMP-9 in monitoring the effect of treatment with chemotherapy and/or radiotherapy on CRC patients. We also evaluated the correlation between plasma levels of NGAL and MMP-9 in CRC patients.

Patients, materials and Methods

Patient population, data collection and therapeutic strategy:

The current study was carried out on 50 diagnosed Egyptian patients with colorectal cancer. Patients were chosen during the period between February 2013 and December 2014. Among cases referred to the National Cancer Institute (NCI), Cairo University, after taking their informed consents. They were 27 (52%) males and 23 (48%) females. Their ages ranged between 21 years and 67 years. Exclusion criteria included pediatric age group, non-Egyptians and patients with double malignancy .Patients with sepsis or severe heart, renal or liver failure were excluded from the study also. Diagnosis of colorectal carcinoma was based on excision biopsy from the affected sites. Histopathological studies were done to confirm the diagnosis .The tumors were classified by grade and stage according to the TNM classification .Full medical history was obtained for all patients through clinical examination and routine laboratory investigations, including liver functions, kidney functions, Hb %, PLTs count and WBCs count. Normal, healthy laboratory volunteers provided their permission verbally. The healthy volunteers had no concomitant illnesses, including no signs of infection, gastrointestinal hepatic or renal disease. The values of the basic laboratory parameters of these participants were within the reference limits. The study was approved by the Institutional Review Board (IRB) of the Egyptian National Cancer Institute.

In addition to surgical intervention adjuvant therapeutic strategies for CRC patients groups (3 and 4) were as follows: 1) One patient with stage I colon cancer received chemotherapy; 2) patients with stage II or III colon cancer routinely received chemotherapy; 3) patients with stage II rectal cancer received radiotherapy; 4) patients with stage III rectal cancer received combined chemotherapy and radiotherapy; 5) patients with stage IV CRC received combined chemotherapy and target therapy. The chemotherapy was based on Folfox, Xelox, Xeloda and Folfiri.

Cases were assigned into 5 groups as followed:

Group 1: Included 30 normal healthy subjects, they were studied as control group. Their ages ranged from 36 to 62 years.

Group 2: Included 15 Patients with early stages (1,I1), who weren't receive chemotherapy and /or radiotherapy .Their ages ranged from 21 to 61 years.

Group 3: Included 15 Patients with early stages (1,11), who were received chemotherapy and/ or radiotherapy . Their ages ranged from 36 to 61 years.

Group 4: Included 10 Patients with late stages (111, 1V), who weren't receive chemotherapy and/or radiotherapy. Their ages ranged from 21 to 65 years.

Group 5: Included 10 Patients with late stages (111, 1V), who were received chemotherapy and/ or radiotherapy. Their ages ranged from 22 to 67 years.

Blood sampling for ELISA, biochemistry and complete blood count study

Serum

Peripheral venous blood samples were collected in vacutainer tubes, allowed to clot for 30 minute at room temperature and centrifuged at 3000 x g for 15 minute. The samples were stored at −20°C until used.

Plasma

Peripheral venous blood samples were collected in heparin tubes for NGAL and MMP-9 determinations allowed to clot for 30 min at room temperature and centrifuged at 1000 x g for 15 min. The samples were stored at −20°C until used.

Whole blood

For determination of Hb %, PLTs count and WBCs count. 1 ml whole blood was collected in plastic tubes containing 0.25 ml EDTA.

Plasma NGAL concentration was measured using the Human Lipocalin-2/NGAL Immunoassay QUANTIKINE® R&D Systems, Catalog Number DLCN20 (Jobs *et al.,* 2014).

Measurement of MMP-9 was performed by R & D systems immunoassays (Human Quantikine ELISA kit, DMP900) (Lewiński *et al.,* 2012).

Statistical analysis

All analyses were done using SPSS software program version 17 (SPSS ,Inc ,Chicago ,III. , USA). Multiple comparisons between groups was done using Bonferroni test. A probability (P) of less than 0.05 was considered statistically significant. The differences among the mean values from all groups were analyzed with one-way ANOVA .The Spearman correlation co-efficient was used to determine the correlation of NGAL with MMP-9.

Results

Plasma NGAL levels in healthy control subjects (G1) ranged from (29 – 110 ng/ml) with a mean value ± SD of [74.9 ± 23.44 ng/ml] , in early stages (I,II) before treatment (not received chemotherapy and /or radiotherapy) patients (G2) ranged from (133 – 257.9 ng/ml) with a mean value ± SD of [183.81 ± 36.81 ng/ml] ,in early stages (I,II) after treatment patients (G3) ranged from (142.4 – 229.1 ng/ml) with a mean value ± SD of [195.06 ± 29.21 ng/ml] ,in late stages (III,IV) before treatment patients (G4) ranged from (150.2 – 331.5 ng/ml) with a mean value ± SD of [198.11 ± 52.83ng/ml] and in late stages after treatment patients (G5) ranged from (178.3 – 277.8ng/ml) with a mean value ± SD of [225.03 ± 32.96 ng/ml].

Statistical analysis revealed that mean plasma NGAL levels in early stages (I,II) before treatment (not received chemotherapy and/ or radiotherapy) patients (G2), early stages (I,II) after treatment patients (G3), late stages (III,IV) before treatment patients (G4) and late stages after treatment patients (G5) increased in comparison to healthy control subjects (G1) and very highly significant at p-value (< 0.0001,< 0.0001, < 0.0001 and <0.0001) respectively Plasma MMP-9 levels in healthy control subjects (G1) ranged from (106 – 477 ng/ml) with a mean value ± SD of [324 ± 92.62 ng/ml] , in early stages (I,II) before treatment (not received chemotherapy and/ or radiotherapy) patients (G2) ranged from (550.3 – 1015 ng/ml) with a mean value ± SD of [853.76 ± 130.77 ng/ml] , in early stages (I;II) after treatment patients (G3) ranged from (770.2 – 1225 ng/ml) with a mean value + SD of [927.36± 133.67 ng/ml] ,in late stages (III,IV) before treatment patients (G4) ranged from (885.6 – 1055 ng/ml) with a mean value ± SD of [935.29 ± 55.49ng/ml] and in late stages after treatment patients (G5) rang-d from (857.5 – 1193.8ng/ml) with a mean value ± SD of [974.07 ± 111.76ng/ml].

Table 1: All Characteristics, Laboratory Findings and Plasma Level of NGAL and MMP-9 in Study Patients and Control Subjects.

Variables		Group 1 (n=30)	Group 2 (n=15)	Group 3 (n=15)	Group 4 (n=10)	Group 5 (n=10)	P value
Age (yr)		36 - 62	21 - 61	36 - 61	21 - 65	22 - 67	
Gender	Male	20	6	9	5	7	
	Female	10	9	6	5	3	
NGAL (ngml)		74.9 ± 23.44	183.81 ± 36.81	195.08 ± 29.21	198.11 ±52.83	225.03 ±32.96	0.0001
MMP-9 (ng/ml)		324 ± 92.62	853.76 ±130.77	927.36 ±133.67	935.29 ± 55.49	974.07 ±111.76	0.0001
AST (IU/L)		21.66± 6.52	24.1± 10.67	29.5 ± 24.3	21.01± 11.94	27.88± 17.3	0.077
ALT (IU/L)		19.1± 7.86	24.3± 17.86	26.06±20.42	19.08± 15.0	21.72± 8.10	0.502
Total Bilirubin(mg/dl)		0.559± 0.22	0.4612±0.10	0.5113±0.228	0.6130± 0.40	0.613± 0.192	0.476
Albumin (g/dl)		3.80± 0.43	3.79± 0.39	3.66±0.42	3.95± 0.34	3.72± 0.37	0.521
Urea (mg/dl)		27.1667± 8.09	24.133± 7.61	33.0 ± 14.65	27.1± 5.89	29.80± 11.29	0.152
Creatinine (mg/dl)		0.84± 0.33	0.77± 0.231	0.9120±0.417	0.7820±0.271	0.8100± 0.417	0.927
Hemoglobin (g/dl)		12.82± 0.8	12.213± 1.7	11.334± 1.458	10.81± 1.58	11.02± 1.664	0.0001
WBC count ($\times 10^3$ /µl)		6.035± 1.5	6.616± 2.9	6.18± 2.482	6.484± 3.3	5.552± 1.455	0.813
PLT count ($\times 10^3$ /µl)		284.73±74.576	295.733± 90.06	253.86± 147.48	391.9±173.306	276.3± 120.17	0.057

Data are expressed as mean ± SD or (n). AST = Aspartate Aminotransferase AST = Alanine aminotransferase. PLT =Platelets. WBC = White Blood Cell

Statistical analysis revealed that plasma MMP-9 mean levels in early stages (I,II) before treatment (not received chemotherapy and/ or radiotherapy) patients (G2), early stages (I,II) after treatment patients (G3), late stages (III,IV) before treatment patients (G4) and late stages after treatment patients (G5) increased in comparison to healthy control subjects (G1) and very highly significant at p-value (<0.0001,< 0.0001,< 0.0001 and <0.0001) respectively.

The correlation between NGAL and MMP9:

The correlation coefficient between NGAL and MMP-9 was 0.286. There was significant positive correlation between NGAL with MMP-9 ($P < 0.05$) (Table 2).

Table 2: The correlation between NGAL and MMP9 in control and different studied groups

			MMP9	NGAL
Spearman's rho	MMP9	Correlation Coefficient	1	286(*)
		Sig. (2-tailed)		0.044
		N	50	50
	NGAL	Correlation Coefficient	286(*)	1
		Sig. (2-tailed)	0.044	
		N	50	50

**Correlation is significant at the 0.05 level (2-tailed).*

Discussion

Colorectal carcinoma (CRC) is one of the most frequent cancers worldwide, and its incidence has been rising because of global population aging (Martı *et al*., 2013).About half of CRCs are detected at advanced stages. Thus the search for new markers is of major interest for early detection of CRC and identification of new potential therapeutic targets (Duvillard *et al*., 2014).

New available laboratory techniques and the need to find better neoplastic markers have challenged us to discover molecules that would help predict tumor extension, recurrence risk, or the need of adjuvant treatment. One of these promising compounds is NGAL, an acute-phase protein with increased production under inflammatory conditions and in the presence of epithelial tumors. Up to now the exact role of NGAL in CRC has remained unclear (Martı *et al*., 2013).NGAL has been recently suggested to take part in the progression of colorectal carcinoma (Barresi *et al*., 2011).

In the present work we examined the levels of NGAL and MMP-9 in 50 colorectal cancer patients with different tumor stages.

Very highly significantly elevated plasma NGAL levels, represented by ($P < 0.0001$) were found in all studied groups compared to control group.

The elevated mean NGAL levels in patients groups may be due to the explanation of Barresi *et al*. reported that NGAL seems to participate in cancerogenesis by favoring iron uptake from extracellular space within the malignant cells, a fundamental process for the maintenance of neoplastic cell multiplication .In line with its involvement in the cancerogenesis processes, NGAL synthesis is induced by factors promoting the development of neoplasias , and its over-expression has been found in several malignancies (Barresi *et al* ., 2011).

Our experimental results are in agreement with those reported by Chang *et al*., (2014) , who found that circulating NGAL levels in both CRC patients who had not received chemo- and/or radio- therapy or had received chemo- and/or radio- therapy before surgery were higher than healthy-control subjects ($P < 0.0001$).

Duvillard *et al*., (2014), reported that higher pre-operative serum concentrations of NGAL (p values < 0.005) were observed in patients with CRC who had not undergone pre-operative radiotherapy or chemotherapy compared to controls.

The study also showed that NGAL mean levels in early stages (I,II) after treatment patients (G3) was elevated compared to early stages (I,II) before treatment (not received chemotherapy and /or radiotherapy) (G2) but statistically insignificant at p-value 1.000 and mean levels in late stages (III,IV) after treatment patients (G5) was elevated compared to late stages (III,IV) before treatment patients (G4) but Statistically insignificant at p-value 0.723.

NGAL expression is regulated by the transcription factors NF-kB, CEBP and others.

Radiation and chemotherapy may induce reactive oxygen species (ROS) that result in NF-kB activation and downstream NGAL transcription. These therapies could result in the synthesis of NGAL in cancer cells, leading to the development of therapy-resistant cells that contribute to the reemergence and metastasis of the cancer; as increased NGAL expression may allow cells to persist under conditions where therapy-sensitive cancer cells could not survive expression (Chappell *et al* ., 2013).

Since the original identification of galetinase B/matrix metalloproteinase (MMP)-9, as a human leukocyte gelatinase , its characterisation as a type V collagenase , the observation that malignant tumour cells express an identical enzyme that associates with metastatic behaviour and degrades type IV collagen under certain conditions (Farina and Mackay, 2014).

In several kinds of cancers, MMP-9 has been reported to be overexpressed, and there has been a great deal of interest of investigating the possible role of MMP-9 in cancer invasion and metastasis. Indeed, high expression levels of MMP-9 have been considered correlated with the survival of cancer patients. Many studies

were published to assess the prognostic role of MMP-9 overexpression in patients with colorectal cancer, but findings from those studies were inconsistent and paradoxical (Li *et al*., 2013).

Very highly significantly elevated plasma MMP-9 concentration, represented by ($P < 0.0001$) was found in all studied groups compared to control group.

Those results may be due to the explanation of Bouchet and Bauvois, (2014). reported that active MMP-9 releases or generates bioactive molecules that in turn bind to specific receptors known to regulate key signalling pathways associated with cell growth, migration, invasion and angiogenesis .For example, MMP-9 can release factors such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 sequestered in the extracellular matrix which stimulate tumour associated-endothelial cells and thus promote angiogenesis and tumor growth. Moreover, MMP-9 sheds and activates pro-tumour necrosis factor (TNF)-α, proTGF-β1 and Kit-ligand which are intimately involved in the regulation of cell growth and angiogenesis. MMP-9 suppresses the proliferation of T lymphocytes through disruption of the IL-2R signalling that may constitute a mechanism of cancer-mediated immunosuppression.

Hadler-Olsen *et al*., (2013), found that in a study of colorectal cancer, the MMP-9 level was significantly higher in the serum from patients with malignant and premalignant lesions compared with the serum from patients with benign lesions.

Our experimental results are in agreement with those reported by Dragutinovic *et al*., (2011) ,who found that MMP-2 and MMP-9 levels were increased in the serum of the patients with colorectal cancer compared to the control group ($P < 0.05$) .

There was a clear difference between plasma levels of MMP-9 in cancer patients at all stages compared to controls, with a significant elevation compared to both normal subjects and those with adenomatous polyps ($p <0.0001$) (Tutton *et al*., 2003).

In this study , we found that MMP-9 mean levels in early stages (I,II) after treatment patients (G3) were elevated compared to early stages (I,II) before treatment (G2) but statistically insignificant at p-value 0.665.and MMP-9 mean levels in late stages (III,IV) after treatment patients (G5) were elevated compared to late stages (III,IV) before treatment patients (G4) but Statistically insignificant at p-value 1.000 .

Treatment with chemotherapy and /or radiotherapy increased the levels of MMP-9 but statistically insignificant. This may be similar to the explanation of Chu *et al*., (2012), reported that neoadjuvant chemotherapy prior to surgery, which has now become standard therapy in many Western countries, has been proved to be able to alter MMP-9 expression. This might be due to the effects of 5-fluorouracil (5-FU) on the activity of nuclear factor-kappaB (NF-jB), which can regulate MMP-9 in human malignancy.

Kumar *et al*., (2000), demonstrated that preoperative high-dose radiotherapy leads to a significant increase in levels of MMP-2 and MMP-9 in rectal cancer tissue, with no increase observed in normal mucosa. And reported that the increased levels MMP-9 in rectal tumour tissue seen after radiotherapy may be due to stromal activation by cytokines produced by the cancer cells in response to the treatment.

We found that late stage groups had higher mean levels of MMP-9 than early stage groups but statistically insignificant.

These results similar to Zhang *et al*., (2012) who reported that among 216 CRC patients, MMP-9 expression in tumor samples was found to be significantly not correlated with TNM stage.

On the other side Dragutinovic *et al*., (2011), showed that there was significant correlation in MMP-9 level among the CRC patients with tumor stage I and II and the patients with tumor stage III and IV.

In this study we found significant positive correlation between the NGAL levels and MMP-9 levels in colorectal cancer. The correlation coefficient between NGAL and MMP-9 was 0.286 and $P < 0.05$.

These findings may be related to the findings of Zhang *et al*., (2009), who found that in rectal cancers, the correlation coefficient between mRNA up-regulation of NGAL and MMP-9 was 0.393. NGAL mRNA up-regulation was positively correlated with MMP-9 ($p < 0.001$).

We can explain this by the findings of Candido *et al* .,(2014) , reported that NGAL's ability to combine in a dimeric complex with MMP-9, results in a protective action of MMP-9 from its auto-degradation and consequently results in a higher gelatinolytic action of MMP-9 on extracellular matrix. By this function, it has been shown that NGAL may promote cancer development in a variety of different cancer types.

Conclusion

Our study showed that

1-NGAL and MMP-9 plasma levels were elevated in colorectal cancer patients with different tumor stages which indicate a potential screening role of them in CRC.

2-The plasma levels of NGAL or MMP-9 not significantly associated with tumor stage or treatment with chemotherapy and /or radiotherapy.

3-our findings support the notion that NGAL and MMP-9 could serve as an molecular markers but not for prognosis for Patients with colorectal cancer , and demonstrated that increased levels of NGAL and MMP-9 in

human CRC have no value in monitoring the effect of treatment with chemotherapy and /or radiotherapy on CRC patients.

4- In this study, there was significant positive correlation between NGAL and MMP-9 in CRC Patients.

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