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ORIGINAL ARTICLE

Considering the relationship between Metalloproteinase activity level of active MMP-2, MMP-9, MMP-9/NGAL, and Dimmer MMP-9 by Grading of Tumor in Colorectal cancer

Helal Tahazadeh[1], Hashem Yaghoubi[1,*], Mohammad Taghi Ahady[1], Hemen Moradi-Sardareh[2]
1 Department of biology, Ardabil Branch, Islamic Azad University, Ardabil, Iran
2 Department of biochemistry, faculty of medicine, Tehran University of medical science, Tehran, Iran
* E.mail: yaghoubi_h@iauardabil.ac.ir

ABSTRACT
A lot of attention of medical science allocated to cancers because of the increasing rate of incidence of cancer in the world wide. Colorectal cancer with a high mortality rate is the second cause of cancer -related deaths. The biopsy and histological examination is need for diagnosis of colorectal cancer and choosing of the best treatment. This method is invasive. In this study, Metalloproteinase's 2, 9, dimmer MMP-9 and NGAL/MMP-9 complex is examined. The purpose of this study was to compare the sensitivity and specificity of serum enzymes in the diagnosis of colorectal cancer. In this study, 33 patients with colorectal cancer and 20 healthy individuals were selected. 10 ml blood of all people collected and serum was isolated. The zymography technique done to check the activities of matrix metalloproteinase. After electrophoresis the gel incubated for 16 hours at Triton 100X then gel were incubated at 37 ° in Zymography buffer, then stained with coomassie blue and finally got bleached. Finally, the results were slightly Total lab software. The activity of four matrix metalloproteinase enzymes was compared between the two groups and the different was significant. The result of comparing enzyme activity of MMP-2 between stages 1 and 2 of colorectal cancer was not significantly different, while between stages 1 and 3 as well as 2 and 3, the difference was significant. Other markers studied were significantly different between the various stages of cancer. The relationship between the staging of colorectal cancer and the activity of MMP-9 and NGAL/MMP-9 was positive and direct, but its relationship with the activity of MMP-2 and MMP-9 dimmer was reverse and negative. The sensitivity and specificity of the activity of MMP-9 and NGAL/MMP-9 for the detection of colorectal cancer were 87 and 85, 66 and 65 %, respectively. Due to the lack of offensive zymography and good results obtained in this study, we concluded that the use of this method to metalloproteinase, especially MMP-9 will be good in the diagnosis and determination of the degree of cancer. Further studies are needed to confirm this statement.
***Keywords**: matrix metalloproteinase 9, matrix metalloproteinase 2, dimmer matrix metalloproteinase 9, NGAL/MMP-9 complex, colorectal cancer, zymography, Iran.*

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death in the United States [1, 2] that allocates 9% of deaths due to cancer to itself. Age over 50 years and family history are the main risk factors for colorectal cancer [3]; other risk factors include nutrition style, overweight, sedimentary life, and smoking [4].

One of the important steps in CRC development is cancer cells metastasis; the metastasis process involves a series of complex cascades which play role in cancer cells migration, attachments and invasion. Cell invasion includes a process of displacement of cancer cells through the extracellular matrix barriers that has been diagnosed as a required major biological event for tumor metastasis [5].

The matrix metalloproteinase is a family of proteolytic enzymes that destroy the extracellular matrix (ECM) components which include collagen, fibronectin, and laminin [6].

The most important enzymes of matrix metalloproteinase are gelatinase A (MMP-2) and gelatinase B (MMP-9) [7,8] that have the most effect on gelatin and that's why they have been called gelatinase [8].
These enzymes digest a wide range of collective tissue in ECM that include collagen type of I, IV, V, VII, X, IX, elastin, fibronectin, aggrecan, vitronectin, and laminin [7]. The gelatinase enzymes can also release and change different factors of angiogenesis and they have role in different pathological cases as cancer, bone diseases, inflammatory disorders, aneurysm, arteriosclerosis, atherosclerosis, and myocardial damages [8].
Matrix metalloproteinase 9 (MMP-9) is a gelatinase with 92 kD molecular weight which breaks down the collagen type IV that is the main part of base membrane structure and extracellular matrix. The previous studies have suggested that MMP-9 is important for regulation of cell migration by TNF-α [9, 10].
It has been determined in a study that inflammation associated with ulcers has increased expression of pro-matrix metalloproteinase 9 and has a key role in colorectal cancer cells development [11].
The matrix metalloproteinase 2 along with matrix metalloproteinase 9, have determined role in differentiation, apoptosis, angiogenesis, immune response, and also cancer cells growth [12]. It has been determined also that the activity level of pro-matrix metalloproteinase 2, active-matrix metalloproteinase 2, and pro-matrix metalloproteinase 9 is noticeable in people stricken with colorectal cancers higher than the controls subjects' mucus. Besides, it was proved by more consideration that the activity level of active-matrix metalloproteinase 2 is a more effective factor for existing invasive cancer than pro-matrix metalloproteinase 2 and pro-matrix metalloproteinase 9 [13].
Neutrophil gelatinase-associated lipocalin (NGAL) glycoprotein is one member of lipocolins family. NGAL due to its association and creation complex with MMP-9 cause more stability of this enzyme. In recent years, NGAL has been known as a biomarker in a series of human diseases and the accomplished studies on cell cultures and mice are representative of NGAL role in the physiological and pathological processes [14].
Complex formation of NGAL with MMP-9 causes prevention of spontaneous degradation of MMP-9, protects of it, increase of its proteolytic activity and also causes accelerating in destruction of ECM [15, 16]; moreover the complex of NGAL/MMP-9 is traceable in the urine of more than 90% of breast cancer patients [15].
The only reliable method for cancer specialists in order to diagnose colorectal cancer and its grading and staging is using of clinical and histological grading results that unfortunately, is totally an invasive method. The activity of MMP-9, MMP-2, Dimmer MMP-9, and NGAL/MMP-9 complex in serum of people stricken with colorectal cancers and control group is considered in this study and then obtained their correlation with cancer pathological stage. The aim of this study is considering these enzymes in colorectal cancer and simultaneously, as a marker at different stages of cancer pointing out their superiority partially according to being non-invasive of the method.

MATERIAL AND METHODS

In this matched case-control study, there were considered 33 people stricken with colorectal cancer at early stages (I, II, III) and 20 people as control group among people who referred to Imam Khomeini hospital, Tehran since September 2014.

Exclusion and inclusion criteria

The people who had been confirmed in them colorectal cancer by surgery and pathology consideration and the people who had not used anti-cancer drugs were included in the study. Those patients whom the duration of stricken with colorectal cancer in them was more than one year, the patients who had done chemotherapy, hormone therapy, and/or radio therapy were excluded from the study.
In the control group, those people whom their colorectal was normal and had no kinds of cancer history were included in the study, and those people who had the history of cancer, anti-cancer drugs consumption, and/or radio therapy were excluded from the study.
Tumor staging was according to the current TNM classification, stage I (T1-2N0M0), II (T3-4N0M0) and III (TxN1-3M0) corresponding to Dukes' stage A, B and C respectively.

Zymography

The serum samples in this method were electrophoresed on SDS-PAGE 10% gel with 1% gelatin (gelatinase substrate), after completion of electrophoresis, gels were incubated for an hour at the room temperature in Triton X100 2% solution, and for 16 hours at 37° in TrisHCI buffer with ph.=7.4 containing 10 mM calcium chloride (Zymography buffer). After extensive washing, gels were painted by G250 Coomassie Blue 5% solution and decolorized by bleaching solution (water 60%, methanol 30%, and acetic acid 10%). Colorless bands that were created in the effect of these enzymes activity MMP-2, MMP-

9, NGAL/MMP-9, and Dimmer MMP-9 appeared in the violet background. Protein weight marker (Sigma Aldrich; USA Color Burst) was used for gelatinase bands authentication.

Inhibition assay

Zymograms of CRC and control groups' serum were incubated with the described zymography buffer, added with EDTA 20mM as inhibitors of MMPs. Following incubation, at 37 C for 18 h, gels were stained with Coomassie blue. After full bleaching the gel scanned by Canon scanner LiDE110, Japan.

Quantification stage of results

Colorless bands of Zymography gels that were created in the effect of the enzymes activity MMP-2, MMP-9, MMP-9/NGAL, and Dimmer MMP-9, quantified by the use of Total Lab TL120 software, by bands area measurement, and by pixels number.

Data analysis

The statistical analysis was used by the use of SPSS V.16 software. The T-test, ANOVA, and Tukey statistical tests used for comparing the mean of obtained results of the enzymes activity MMP-2, MMP-9, MMP-9/NGAL, and Dimmer MMP-9 in patients and control groups and the results were reported according to Mean±SD. In addition, there were used of bivariate correlation test and Spearman's rho statistical method for considering the relationship between types of markers activity and cancer stage. All reported results with (CI: 95%) and p-Value <0.05 were considered as significant difference.

RESULTS

The demographic result of age, weight, height, gender, and BMI were compared between two control and cancer groups by the use of T-test statistical test; the results have been shown at the Table 1.

Table 1: Comparison of demographic result between two groups.

variable	Group	N	Mean	Std. Deviation	Std. Error Mean	P value
weight	CRC	33	73.0303	14.54408	2.53180	0.260
	control	20	69.3000	9.29686	2.07884	
age	CRC	33	60.4848	14.49811	2.52380	0.850
	control	20	59.7000	14.67938	3.28241	
height	CRC	33	165.2121	8.17679	1.42340	0.219
	control	20	162.0500	10.17466	2.27512	
BMI	CRC	33	26.8018	5.23580	.91144	0.948
	control	20	26.7056	5.18664	1.15977	
Gender	CRC	33	1.4848	.50752	.08835	0.917
	control	20	1.5000	.51299	.11471	

CRC: Colorectal cancer; Std: Standard; N: number of case.

There were no statistical differences between two groups in age, weight, height, BMI and gender, so we considered that two groups are matched.

Zymography gel results

At first, the existences of all markers diagnosed by Gel Zymography and by molecular weight marker (figure 1) then quantified and analyzed the obtained results of Zymography gel (Table 2).

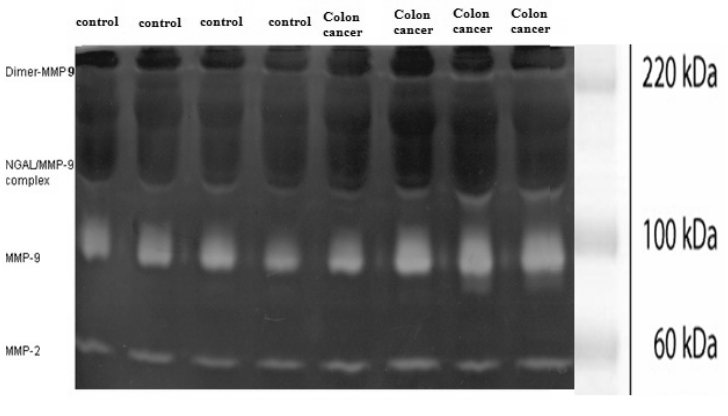

Figure 1: The existences of various MMPs were diagnosed in their weight range by the use of weight marker in CRC groups and healthy individuals.

The comparison of different markers between the two studied groups has been taken at the Table 2.

Table 2: Quantified activity level of MMPs in colorectal cancer patients and healthy individuals.

Markers	Group	N	Mean	Std. Deviation	Std. Error Mean	P value
Dimmer MMP-9	CRC	33	163.6371	27.36190	4.76310	0.000
	control	20	94.3092	72.97917	16.31864	
MMP-9/NGAL	CRC	33	55.7697	46.83318	8.15261	0.001
	control	20	18.0633	7.25436	1.62212	
MMP-9	CRC	33	352.8148	93.87292	16.34118	0.000
	control	20	193.5710	40.17544	8.98350	
MMP-2	CRC	33	130.5064	44.27224	7.70681	0.000
	control	20	70.9011	24.46196	5.46986	

CRC: Colorectal cancer; Std: Standard; N: number of case.

Different markers of MMP-2, MMP-9, Dimmer MMP-9, and the complex of NGAL/MMP-9 were compared together two by two by Tukey test; the results have been taken at the Table 3.

Table 3: comparison of enzymes activity in different stage of cancer by Tukey test.

Dependent Variable	(I) stage of cancer	(J) stage of cancer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
MMP-2	1.00	2.00	.24549	1.07455	.972	-2.2733	2.7642
		3.00	6.47576*	1.10840	.000	3.8777	9.0739
	2.00	1.00	-.24549	1.07455	.972	-2.7642	2.2733
		3.00	6.23027*	.95285	.000	3.9968	8.4638
	3.00	1.00	-6.47576*	1.10840	.000	-9.0739	-3.8777
		2.00	-6.23027*	.95285	.000	-8.4638	-3.9968
MMP-9	1.00	2.00	-105.96917*	.86138	.000	-107.9883	-103.9501
		3.00	-192.50416*	.88852	.000	-194.5869	-190.4215
	2.00	1.00	105.96917*	.86138	.000	103.9501	107.9883
		3.00	-86.53499*	.76383	.000	-88.3254	-84.7446
	3.00	1.00	192.50416*	.88852	.000	190.4215	194.5869
		2.00	86.53499*	.76383	.000	84.7446	88.3254
Dimmer MMP-9	1.00	2.00	-10.18419*	.61914	.000	-11.6355	-8.7329
		3.00	12.95063*	.63865	.000	11.4536	14.4476
	2.00	1.00	10.18419*	.61914	.000	8.7329	11.6355
		3.00	23.13482*	.54902	.000	21.8479	24.4217
	3.00	1.00	-12.95063*	.63865	.000	-14.4476	-11.4536
		2.00	-23.13482*	.54902	.000	-24.4217	-21.8479
MMP-9/NGAL	1.00	2.00	-61.54305*	.94369	.000	-63.7550	-59.3310
		3.00	-19.92380*	.97342	.000	-22.2055	-17.6421
	2.00	1.00	61.54305*	.94369	.000	59.3310	63.7550
		3.00	41.61924*	.83681	.000	39.6578	43.5807
	3.00	1.00	19.92380*	.97342	.000	17.6421	22.2055
		2.00	-41.61924*	.83681	.000	-43.5807	-39.6578

*. The mean difference is significant at the 0.05 level.

The bivariate correlation test and Spearman's rho statistical method were used for considering the relationship between cancer stage and types of markers activity change (Table 4). It is determined in this test that which marker has relationship with cancer stage and whether their relationship is direct or reverse.

Table 4: the correlation of markers activity and stage of cancer by Spearman's rho

		Stage of cancer	Dimmer MMP-9	MMP-2	MMP-9	NGAL/MMP-9
Stage of cancer	Correlation Coefficient	1.000	-.271**	-.037**	.935**	.115**
	Sig. (2-tailed)	.	0.000	0.000	.000	.000
	N	11644	11644	11644	11644	11644

**. Correlation is significant at the 0.01 level (2-tailed).

Receiver operating characteristic (ROC)

Different markers sensitivity and specificity were determined by ROC curve. Because it was determined in the above analysis that there is no relationship between cancer stage with MMP-2 and Dimmer MMP-9, therefore, there has been drawn ROC curve only for MMP-9 and NGAL/MMP- 9 complexes (Figure 2 and Table 5).

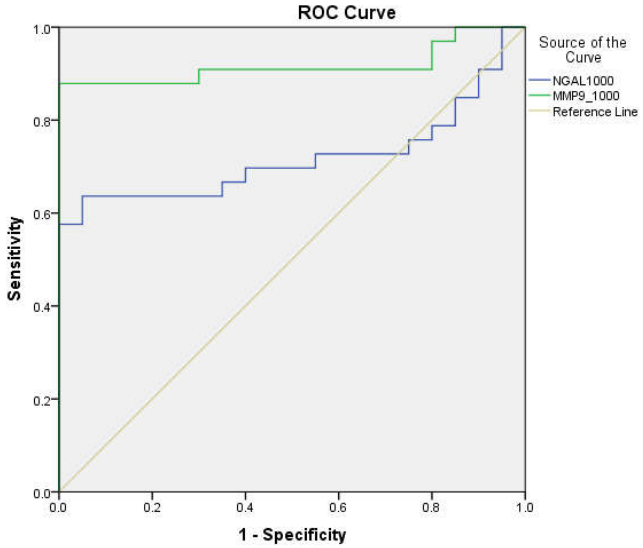

Figure 2: ROC curve for MMP-9 and MMP-9/NGAL complex markers

Table 5: Area Under the Curve(AUC) of ROC

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
MMP-9NGAL	.718	.072	.008	.578	.858
MMP-9	.917	.043	.000	.833	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

DISCUSSION AND CONCLUSION

According to the accomplished studies through the world and Iran, it has been determined that the activity of different matrix metalloproteinase changes in colorectal cancer. It was compared in this study the obtained results of Zymography method with sampling gold standard method by colonoscopy to report that if it is possible to use this method to determine the degree of cancer in a non-invasive and at short time.

The comparison of the MMP-9, Dimmer MMP-9, and MMP-9/NGAL complex activity between two control and cancer groups had significant difference (P value<0.001) in this study and these results were in line with Davies et al. results in which they concluded that the MMP-9 levels in control group were significantly lower than bladder cancer group and MMP-9 level significantly increased by enhanced cancer stage in cancer patients [17].

The comparison of obtained results of MMP-2 activity was significant (P value < 0.001) in the present study, and this result was in line with obtained results of Marwan et al. study that perceived MMP-2 serum values in stomach and colorectal cancer patients were averagely higher than control people, but were not significant [18]. Also, in a study that Davies et al conducted, they perceived MMP-2 active values were higher in cancer group than control, but had not reached at significant level [17].

The present study by considering the activity of MMP-2 marker among different stages of cancer in people stricken with colorectal cancer showed that this marker activity had not significant difference between 1 and 2 stages of colorectal cancer (P value<0.972), while other markers activity indicated significant differences between different stages of cancer. This result is consistent with Murnane et al results that reported the active MMP-2 activity increased during the progression of colorectal adenoma towards control group people [13].

In this study, different stages of cancer had low and reverse correlation with the activity of Dimmer MMP-9 and active MMP-2 enzymes, but their correlation with the activity of NGAL/MMP-9 complex was low

and direct, while different stages of cancer correlation with MMP-9 was very positive and direct. Our results appear to contrast with the findings reported by Marwan et al. that perceived no relationship between MMP-9 values and clinical and histological grading [18], but Our data are compatible with the study results of Baker and Leaper [19] that perceived MMP-9 values had relationship with Dukes grading and colorectal cancer lymphatic invasion. Bogusiewicz *et al* there was seen no relationship between MMP-9 activity and clinical grading or tumor size [20].

Although the relationship has been contradicted between MMP-9 serum values and clinical and/or histological grading in different studies, it will be suggested that this may be related to different methods of study and the increase of MMP-9 level may have relation with tumor progression.

The MMP-2 level in the present study had converse relationship with disease stage, but neither Libakk et al nor Parsons et al had perceived such relationship [21, 22], while in Was study the activity level of active forms and pro-MMP-2 in patients with cancer in stage IV were very lower than people whom their cancer was localize and had not given metastasis [23]. These results suggest the MMPs activity might be different in separate stages of a disease because there are different stroma components around tumor [21].

The significant increase of NGAL/MMP-9 complex in both stomach and colorectal cancers has been seen in different studies [24, 25], and this is probably reflexive of their biological importance in transforming of tumor from benign to malignant and probably shows their role in tumor progression.

In a study that Marwan et al conducted, there was seen no significant relationship between NGAL/MMP-9 complex serum level and clinical and histological grading in both stomach and colorectal cancer patients [18].

The sensitivity and specificity of the activity of MMP-9 and NGAL/MMP-9 enzymes were measured by the use of ROC curve and getting cut-off point in the present study; for MMP-9 activity in this study with cut-off point about 250 AU the sensitivity and specificity were 87% and 85% respectively, and for MMP-9/NGAL complex marker with cut-off point about 20 AU were obtained 66% and 65% respectively.

In a study that Murnane et al conducted on the activity of colon active MMP-2 in different groups, it was determined that MMP-2 could diagnose cancer with 79% sensitivity and 69% specificity (13).

It is concluded from this study that considering MMP-9 activity in control and cancer people can be somewhat used as replacement for other markers, but this subject must be considered in other studies for final approval and the sample size be furthered, too. In addition, this marker has shown it can be used for tumor stage estimation which for approval of this subject, besides increasing the sample size and considering it in other cancers, this subject must be put beside other diagnostic methods and then reach final decision.

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CONFLICT OF INTEREST

Declare that there is no conflict of interest regarding the publication of this article.

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