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Correlation between cystatin-C, acute phase reactants, and retinopathy severity in diabetic patients

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Received: 2017-04-29 Accepted: 2017-12-25

糖尿病患者血清胱抑素 C 和急性反应指标与视网膜病变严重程度相关性研究

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摘要

目的:评估糖尿病视网膜病变(DR)严重程度与血清胱抑素 C 和急性时相反应指标的相关性,包括红细胞沉降率(ESR)和超敏 C 反应蛋白(hs-CRP)。

方法:研究纳入了 1mo 内就诊的所有糖尿病视网膜病患者。患者均记录人口统计学数据。行眼科检查,同时检测糖化血红蛋白(HbA1c)、ESR、hs-CRP 和血清胱抑素 C 水平。

结果:研究包括 67 例糖尿病患者,其中 19 例(28.3%)无视网膜病变患者,22 例(32.8%)非增殖型视网膜病变患者和 26 例(38.8%)增殖型视网膜病变患者。三组间平均年龄、性别分布、平均糖尿病病程、高血压和血脂异常患病率、吸烟状况以及 HbA1c 水平无明显差异。随着视网膜病变发展,平均血清胱抑素 C 水平显著提高,三组分别为 1.1±0.48,1.22±0.38,1.71±0.92 (P=0.007)。在多元回归分析中,仅血清胱抑素 C 与糖尿病视网膜病变严重程度有关(P=0.025)。

结论:研究表明,在独立于急性时相反应指标,血清胱抑素 C 水平随 DR 加重升高。因此,该结论可作为初级医护人员区分高危患者的标志。

关键词:血清胱抑素 C;红细胞沉降率;超敏 C 反应蛋白;糖尿病视网膜病变;炎症反应标志物;急性时相反应物

引用:Khadamy J, Yaghoobi G, Rad AH, Najafi M, Heydari SR, Khademi P. 糖尿病患者血清胱抑素 C 和急性反应指标与视网膜病变严重程度相关性研究. 国际眼科杂志 2018;18(3):412-417

Abstract

• AIM: To evaluate correlation of cystatin-C (Cys-C) with severity of diabetic retinopathy (DR) and acute phase reactants, including erythrocyte sedimentation rate (ESR) and high-sensitivity C-reactive protein (hs-CRP).

• METHODS: All diabetic patients who were referred for diabetic retinopathy (DR) screening during 1mo were enrolled. Demographic data were recorded. All patients have undergone full ophthalmic exam. At the same day, all patients were tested for hemoglobin A1c (HbA1c), ESR, hs-CRP, and Cys-C serum levels.

• RESULTS: Sixty seven diabetics were enrolled, including 19 (28.3%) without retinopathy, 22 (32.8%) non-proliferative retinopathy, and 26 (38.8%) proliferative retinopathy patients. The mean age, sex distribution, mean duration of diabetes, prevalence of hypertension and dyslipidemia, smoking status and HbA1c levels were not significantly different among the three groups. The mean levels of Cys-C increase significantly as retinopathy progress [1.1±0.48; 1.22±0.38; 1.71±0.92 (P=0.007), respectively]. In multiple regression analysis, just Cys-C was significantly associated with severity of DR (P=0.025).

• CONCLUSIONS: This study revealed that serum levels of Cys-C increase while DR progress independently of acute phase reactants. Therefore, it could be used as an associated marker by primary care physicians to distinguish patients at higher risk of severe DR. Larger randomized studies are warranted to confirm findings. Reviewing physiological role of the Cys-C, we proposed that the Cys-C may be a protective response to catalytic stress rather than being a pathogenic factor in microangiopathies.

• KEYWORDS: cystatin-C; erythrocyte sedimentation rate; high-sensitivity C-reactive protein; diabetic retinopathy; inflammatory markers; acute phase reactants
DOI:10.3980/j.issn.1672-5123.2018.3.03

Citation: Khadamy J, Yaghoobi G, Rad AH, Najafi M, Heydari SR, Khademi P. Correlation between cystatin-C, acute phase reactants, and retinopathy severity in diabetic patients. *Guoji Yanke Zazhi*(*Int Eye Sci*) 2018;18(3):412-417

INTRODUCTION

Diabetic retinopathy (DR) is a microvascular comorbidity of diabetes mellitus (DM). Current guideline for diabetic retinopathy screening in DM type 1 is mainly based on duration of disease but it has been reported that hemoglobin A1c (HbA1c) and duration of disease can only predict 11% of patients with DR[1]. To adjust screening schedules, investigation of further associated risk factors are necessitated. It is not possible to fully predict, prevent, and treat DR until we know the role of all factors in pathophysiology of the disease. The pathogenesis of DR is not fully understood but it is believed that complex metabolic dysfunction, oxidative damages, and inflammatory mechanisms may contribute to it[2-6]. To find new associated factors of DR, one may explore suggested risk factors of other microvasculopathies in DM. Diabetic retinopathy and nephropathy are co-findings in many diabetics, common contributing factors may explain this concordance[7].

In recent years, several clinical studies have investigated serum levels of cystatin-C (Cys-C) in various diseases[8-16]. Cys-C is an endogenous cysteine proteinase inhibitor which is produced by all nucleated cells. Its serum concentration is minimally dependent on protein intake and body mass. It is mainly catabolized by the kidneys[17]. It found to be superior to serum creatinine in distinguishing impaired kidney function and a strong predictor of diabetic nephropathy (DN). Moreover, it could predict DN earlier even in pre-diabetic state[8]. In addition, it is associated with other vasculopathies such as diabetic foot[9] and cardiovascular disease[10]. Few studies have assessed correlation of the Cys-C levels with severity grading of DR which revealed that its serum level is related to severity of DR[12-16]. Not only Cys-C is elevated in those with DR[14] but also normal populations with elevated Cys-C levels found to be at greater risk of DR[12]. Those with higher Cys-C levels reported to progress to more severe DR[14]. Altunoğlu *et al*[11] found a correlation between Cys-C levels and acute-phase reactants, including erythrocyte sedimentation rate (ESR) and CRP in non-diabetic nephropathy. Sun *et al*[16] speculated that higher serum Cys-C may lead to inflammation thus may increase CRP. Correlations of Cys-C levels with ESR and high-sensitivity C-reactive protein (hs-CRP) have not been reported yet in patients with DR.

In this study, the association of Cys-C levels to acute phase reactants (ESR and hs-CRP, as general inflammatory markers) and severity of DR in diabetics without DN have been investigated. Furthermore, physiological roles of Cys-C were reviewed.

SUBJECTS AND METHODS

Study Subjects, Area, and Design All patients with type 2 DM who were referred for DR screening to the eye clinic of Vali-e-Asr Hospital (Birjand City, South Khorasan, Iran) during 1mo (September 2015) were recruited. Exclusion criteria included failure to obtain consent, presence of any systemic disease other than DM including chronic kidney disease (CKD), inflammatory and collagen vascular diseases, and any history of surgery including eye surgery. Patients with DN also have been excluded. These were assessed by history taking and reviewing their past laboratory information.

Past medical history of patients was recorded, including age, sex, duration of diabetes, history of hypertension (HTN), history of smoking, and lipid profile. The study was adherent to the principles of the Declaration of Helsinki and approved by ethical committee of ophthalmology department.

Ophthalmic Exam All patients have undergone full dilated ophthalmoscopy by two examiners to grade DR based on International Clinical Disease Severity Scale[18]. In case of any discrepancy, patients were re-examined by either of ophthalmologists to resolve it.

Collection and Processing of Blood Samples At the same day of exam, venous blood samples of all patients were collected from antecubital fossa and tested for ESR (by Westergren method), hs-CRP (by Hitachi 7080; TIA method), Cys-C (by immunoturbidometry method), and HbA1c levels.

Statistical Analysis The SPSS software (version 16, SPSS, Inc., Chicago, IL, USA) was utilized in data analysis. One-way ANOVA and Kruskal-Wallis h-tests were used to examine parametric and nonparametric variables, respectively. Bivariate correlation analysis of Spearman was performed to find any correlation between Cys-C, ESR, hs-CRP, HbA1c, and duration of disease. Multiple regression analysis was performed to find if any of factors including gender, age, duration of disease, HbA1c, Cys-C, ESR, hs-CRP, smoking state, dyslipidemia, and HTN history are independently associated to severity of DR($P<0.05$).

RESULTS

In this prospective study, a total of 67 diabetic patients were enrolled. The study groups included 19 (28.3%) patients with no apparent DR (NDR), 22 (32.8%) patients with non-proliferative DR (NPDR), and 26 (38.8%) patients with proliferative DR (PDR). The sex distribution ($P=0.18$), mean age ($P=0.13$), duration of DM after diagnosis ($P=0.17$), HbA1c levels ($P=0.44$), prevalence of history of HTN ($P=0.96$), prevalence of dyslipidemia ($P=0.36$) and prevalence of positive smoking history ($P=0.22$) were not significantly different among the three groups. Table 1 summarizes demographic information of the three study groups.

Table 1 Demographic information of study groups

Variable	NDR	NPDR	PDR	*P*
Patients	19	22	26	
Age (y)	54.47±9.5	59.18±10.09	59.54±7.38	0.13
Sex (F/M)	10/9	6/16	13/13	0.18
Duration (y)	8.45±4.47	11.65±5.96	11.60±5.05	0.17
Hypertension (%)	57.89	59.09	61.53	0.96
Dyslipidemia (%)	63.15	40.9	53.84	0.36
Smoking (%)	26.31	31.81	11.53	0.22
HbA1c (%)	7.33±0.44	7.25±0.8	7.08±0.7	0.44

NDR: No apparent diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; HbA1c: Hemoglobin A1c; Categorical data are presented as *n* (%); Continuous data are presented as mean±SD deviation.

Table 2 Cys-C, ESR, and hs-CRP serum levels in the study groups

Variables	NDR	NPDR	PDR	*P*
Cys-C (mg/L)	1.1±0.48	1.22±0.38	1.71±0.92	0.007
ESR (mm/h)	13.37±7.04	13.09±9.25	20.08±18.68	0.13
hs-CRP (mg/mL)	9.05±9.7	7.73±9.28	7.58±4.86	0.81

Cys-C: Cystatin-C; ESR: Erythrocyte sedimentation rate; hs-CRP: High-sensitivity C-reactive protein; NDR: No apparent diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; $P<0.05$ (significant differences between the three groups).

Table 3 The bivariate correlation of Cys-C, ESR, hs-CRP, HbA1c, and duration of diabetes mellitus

Variables	Cys-C (mg/L)	ESR (mm/h)	hs-CRP (mg/mL)	HbA1c (%)	Duration (y)
Cys-C (mg/L)	-	<0.001	0.13	0.72	0.84
ESR (mm/h)	<0.001	-	0.004	0.41	0.85
hs- CRP (mg/mL)	0.13	0.004	-	0.9	0.86

CYS-C: Cystatin-C; ESR: Erythrocyte sedimentation rate; hs-CRP: High-sensitivity C-reactive protein; HbA1c: Hemoglobin A1c; All values are *P* of two-tailed significance; Analyzed by Pearson's bivariate correlation test; $P<0.05$ considered significant.

Although the study demonstrated no significant difference in terms of mean acute phase reactants levels (ESR, $P=0.13$; hs-CRP, $P=0.81$) levels, there were significant differences in mean levels of Cys-C ($P=0.007$) among the groups. Table 2 compares study groups in terms of mean ESR, hs-CRP, and Cys-C levels.

In Bivariate(Spearman) correlation analysis (Table 3), there was a correlation between Cys-C and ESR ($P<0.001$) but neither hs-CRP ($P=0.13$) nor HbA1c ($P=0.72$) and duration of disease ($P=0.84$).

A multiple regression analysis was run to find factors associated to severity of DR from gender, age, duration of disease, HbA1c, Cys-C, ESR, hs-CRP, smoking state, dyslipidemia, and HTN history. Although these variables are marginally statistically significantly associated to severity of DR [$F(10, 57)=1.942$, $P=0.058$] with an R^2 of 0.255, but only Cys-C found to be an independent associated factor ($P=0.025$).

DISCUSSION

Results of current study are in line with previous reports concerning that Cys-C levels are correlated to severity of DR[12-16]. Cys-C levels in normal population under and over 50y of age are in range of 0.53-0.92 and 0.58-1.02 mg/L, respectively[19]. The mean Cys-C levels in all three groups of this study were above the normal levels (>0.48±1.1 mg/L; range: 0.4-3.91 mg/L). The mean Cys-C levels in PDR group was 0.92±1.71 mg/L. He *et al*[14] also reported levels greater than 1.25 mg/L in severe NPDR and PDR. They found that Cys-C levels greater than 1.25 mg/L increase the risk of severe DR by 11-fold. They suggested that Cys-C levels could be utilized as an efficient and inexpensive screening index to predict the sight-threatening diabetic retinopathy (STDR). They recommended that it may decrease rising financial burden of DR screening.

There is no earlier report demonstrating that Cys-C in DR is correlated to ESR, hs-CRP, or inflammatory markers. In this study, Cys-C levels found to be correlated to ESR but not hs-CRP or HbA1c levels. In multiple regression analysis, Cys-C was the sole independent risk factor of DR. Font *et al*[20] also reported that Cys-C levels are not associated with inflammatory markers (CRP, interleukin-6, and fibrinogen) in non-diabetic patients with CKD. It should be emphasized that many factors may influence Cys-C levels, including age, sex, height, weight, smoking status, HTN, cardiovascular diseases, and inflammatory conditions beyond the kidney function[21-22]. For an instance, ectatic vascular diseases are correlated with lower Cys-C levels but coronary artery disease is correlated with higher levels of Cys-C[21-22]. While interpreting the results, one should consider these confounding factors which may influence the significance of

findings.

In our series, there was no association betweenthe ESR levels and DR severity. In bivariate analysis, the ESR levels were correlated to Cys-C and hs-CRP, but not HbA1c levels. The ESR levels were not found as independent risk factor of DR severity in multivariate regression analysis. The correlation of the ESR and DR has yet to be fully published. Although serum concentrations of Interleukin (IL)-1beta, tumor necrosis factor (TNF)-alpha, and vascular endothelial growth factor (VEGF) as contributing factors of DR development found to be correlated to ESR[23], But it has been reported that there is no difference between diabetics with and without DR and normal population in case of ESR levels[24]. The ESR is non-specific inflammatory marker which is affected by any factors that alters adhesiveness and aggregation of red blood cells (RBC). These are affected by many other factors such as immunoglobulin, CRP, cholesterol, triglycerides, albumin, high fibrinogen levels, and also anemia[25]. To fully investigate the correlation of ESR and DR, larger studies are warranted.

Although some studies found that higher CRP levels may be negatively correlated to DR[26], but in some other studies hs-CRP levels was positively related to DR and it was suggested to be correlated to vascular complications[27]. In a recent meta-analysis, it was found that the CRP levels might be used as a biomarker to determine the severity of DR[28]. Remarkably, metabolic control (reduced HbA1c, fasting blood glucose, and triglyceride) can decrease the hs-CRP levels. Interestingly, PDR group in this study had a better metabolic control which was reflected by having lower HbA1c than the other two groups. This could be justified by better control among patients when severe complications of DM initiate to show off and put patients on alarm. In current series, there was no association between hs-CRP and Cys-C levels nor severity of DR. Better control among patients with more severe disease, may explain why no correlation between grade of hs-CRP and neither DR nor Cys-C was found. The hs-CRP levels were affected by sex, age, ethnicity, weight, DM type, hyperglycemic control drugs type, glycemic control, lipid profile, smoking history, hormone replacement therapy, and various medications[28-30]. Any discrepancy between the three groups in matter of these confounding factors may influence the repeatability of results of the study.

There were evidences that retinal pigment epithelium (RPE) secrets Cys-C which previously thought to play a role in blood vessel wall disintegrity, inflammation, neurodegeneration, neovascularization process[8], and macular degenration[31]. Domingueti *et al*[15] found that endothelial dysfunction and hypercoagulability biomarkers are in association with Cys-C levels in DM type 1 patients with DR. Therefore, it was speculated that accumulation of Cys-C in endothelial cells may play a role in DR pathogenesis. Sun *et al*[16] proposed that higher Cys-C levels in serum could be taken up into the retinal pigment layer and interfere with endothelial pump activity which may lead to inflammation and CRP rise. These were contrary to findings of current study that there was no association between Cys-C and CRP. Although there were large cohorts that reported a correlation between inflammation status and Cys-C[22-33], but there were recent reports that CRP levels were not correlated to Cys-C levels in important diseases[34-35]. Interestingly, recent studies also challenged this correlation and supports finding of current study, they demonstrated that not only Cys-C is not correlated to inflammatory status of patients but also it is a marker of successful aging[34,36].

It has been found that lower levels of Cys-C or defective mutant Cys-C is correlated with hereditary cystatin-C amyloid angiopathy, age related macular degeneration, atherosclerosis, abdominal aortic aneurysm, Alzheimer's disease and poorer prognosis in breast cancer. These were justified by either amyloid formation or increased proteinase activity. Moreover, Cys-C has demonstrated to have protective effects against various oxidative stresses that induce cell death[37]. Cysteine protease inhibitors also suggested to be used for treatment of neurodegenerative diseases[38]. Hence, Cys-C may have a protective rather than harmful role. Therefore, we reviewed the physiological tasks of Cys-C to propose a new role for it in pathogenesis of DR.

There is an association between insulin resistance and protein oxidative stress which leads to formation of protein oxidation products then inflammation and overproduction of resistin, TNF-α, and IL-6[39]. In diabetics, carbonylated proteins levels were elevated which were vulnerable to proteolysis and the markers of oxidative stress. Cys-C inhibits cysteine protease which is remarkable enzymes in proteolysis and apoptosis[40]. Cys-C expression is stimulated by stress may play a role in inhibitory regulation of apoptosis in oxidative stress[41]. Furthermore, Cys-C reduces the expression and release of IL-1β and TNF-α[42]. These findings could be translated to this: Cys-C inhibits proteolysis and apoptosis, so decrease the possible inflammatory response. Elevated Cys-C in diabetics could be a protective response of stressed cells rather than being interpreted as harmful accumulates which were absorbed or overproduced by the RPE cells in DR[16]. It could be used as a biomarker which is related to severity of catalytic stress but as a response to it, not as a contributing factor. In other words, an optimal concentration of Cys-C may protect cells but if higher concentrations could be toxic is unknown[43]. It is in contrast to previous proposals which introduced the Cys-C as a contributing factor in DR pathogenesis[15-16]. Obviously, findings of current study should be confirmed by further larger studies. The main role of Cys-C in pathogenesis of DR is not fully understood. To elucidate the role of it in stressed cells, we suggested that further studies to focus on association of Cys-C levels with catalytic and oxidative products. In this scope, assessment of correlation of Cys-C to levels of malondialdehyde (MDA), advanced glycation end-products (AGEs), their receptors (RAGE), soluble RAGE (sRAGE), and ferric reducing ability of plasma (FRAP) is suggested[44]. If the Cys-C or

other cysteine protease inhibitors could be used as therapeutic agents in DM remains to be investigated.

Findings of this study were limited by small size of study, no healthy control enrolment, single center design of study (however the center is the only ophthalmic center of region), blood pressure (BP) levels of subjects also has not been analyzed (there is a relation between BP, DR, and also Cys-C in diabetics[45]), creatinine levels of subjects has not been analyzed (however none of subject had DN), body mass index (BMI) of subjects has not been analyzed (Cys-C is moderately correlated with weight and weakly correlated with height) and other inflammatory factors (*e. g.* IL-1beta, IL-6, TNF-α, VEGF, Von Willebrand factor, ADAMTS13, and d-Dimer) has not been investigated. Despite these limitations, it is the first study that has investigated association of Cys-C with ESR, hs-CRP, and DR in diabetic patients who had not DN. Advances in understanding the role of Cys-C in development of DR may improve screening, diagnosis, prediction, follow up, and treatment of retinopathy. Cys-C use in routine practice was limited by lack of cut-off values, large number of clinical studies, guidelines on indications of the test, and the cost-benefit balance of the test[8].

In summary, current study confirms findings of few available studies that Cys-C levels were correlated with severity of DR. The Cys-C was found to be risk factor of DR independently of HbA1c, ESR, hs-CRP levels, and duration of disease. We proposed that rise of Cys-C may be a protective response to catalytic stress which diabetics are in. It is in contrast to previous proposals that put forward a harming effect of accumulated Cys-C on endothelial pump activity[16]. Confirmation of these findings merits further larger studies. It is also recommended that intravitreal level of Cys-C to be measured in future, to elucidate if its level is corresponding to systemic serum level or it is overproduced locally in eyes with DR. Furthermore, we suggested that future studies to evaluate association of Cys-C levels with MDA, AGEs, RAGE, sRAGE, and FRAP[44].

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