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The Relationship between Serum and Gene Expression Levels of RANK, RANKL and Osteoprotegerin Inflammatory Pathway with Unstable Angina: A Case-control Study

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ABSTRACT

Osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK) and receptor activator of nuclear factor-kappa B ligand (RANKL), the members of the tumor necrosis factor (TNF) family, have multiple effects on bone metabolism, endocrine functions and, as an inflammatory pathway, in the immune system. This study tried to determine the association of the OPG/RANKL/RANK axis with the severity of unstable angina (UA) as an inflammatory condition.

Our study involved 50 patients with UA and 50 healthy people. Serum and peripheral blood mononuclear cells were isolated from all participants. Serum levels and gene expression of OPG, RANKL, and RANK in mononuclear cells were measured by enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR), respectively. For each patient with UA, the thrombolysis in myocardial infarction (TIMI) and the global registry of acute coronary events (GRACE) scores were determined to evaluate the severity of the disease. Then we analyzed the relation of OPG, RANKL, and RANK levels with TIMI and GRACE scores in patients with UA. Discriminate analysis was used to predict the combinational models of such factors on the prediction of UA.

Serum levels of OPG and RANKL ($p<0.001$) and gene expression of RANKL ($p<0.001$) were significantly more in patients than those in healthy ones. No relation was seen between the OPG/RANKL/RANK axis and the severity of UA according to TIMI and GRACE scores.

Our study shows that serum level, as well as gene expression of OPG/RANKL/RANK axis neither, predicts the occurrence of UA nor shows any relationship with its severity.

Keywords: Osteoprotegerin; Receptor activator of nuclear factor-kappa B; RANK ligand; Unstable angina

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INTRODUCTION

Unstable angina (UA) is one of the most common forms of coronary artery disease in which mismatch between demand and supply occurs mainly due to atherogenesis (fatty buildups) and plaque destabilization, as well as rupture, leading to thrombus formation.[1] Several inflammatory mediators play some roles in such pathogenesis[2,3,4,5] such as c-reactive protein (CRP)[2], interleukin (IL)-2,[4] and IL-6.[2] Some new studies have also shown increased levels of IL-37 and IL-18 in acute coronary syndrome.[5] Although receptor activator of nuclear factor-kappa B ligand (RANKL) (receptor activator of nuclear factor kappa-B ligand) and its ligands, namely osteoprotegerin (OPG) (soluble decoy receptor) and receptor activator of nuclear factor-kappa B (RANK) (membrane-bound receptor) as members of the tumor necrosis factor (TNF) family have multiple effects on bone metabolism and endocrine system, they also have some effects on the immune system as inflammatory mediators.[6,7,8] In bone metabolism, RANKL plays an essential role in the activation of osteoclasts and which are responsible for bone resorption.[9] The interaction between RANKL and RANK induces both osteoclastogenesis and activation of osteoclasts.[10] OPG that is known as an osteoclast inhibitor factor, in competition with RANK is connected to RANKL and restricts RANK activity at osteoclastogenesis and bone resorption; and in this way, the OPG–RANKL complex plays a key role in bone homeostasis.[7]

RANKL and OPG are produced by several tissues including the kidney, intestine, lung, and bone.[11] This indicates that they are not organ-specific markers. In the inflammatory context of atherosclerosis, both endothelial and vascular smooth muscle cells produce RANKL[12] and OPG.[13] Such production is up-regulated by pro-inflammatory cytokines produced by monocytes and lymphocytes that have been recruited into the intima of the atherosclerotic vessel wall.[12,14,15] In the atherosclerotic plaques, RANKL binds to OPG which has two paradoxical effects. From one side it increases the activity of matrix metalloproteinase which degrades the extracellular matrix and reduces the thickness of the fibrous cap. Such erosion leads to plaque rupture and thrombus formation promoting osteogenesis which consequently leads to the synthesis of some kind of bone proteins and matrix calcification within the arterial vessel.[12] And from the other side, they modulate the release of matrix-degrading enzymes such as cathepsins and in this way might influence plaque vulnerability.[16,17] Such a mechanism may explain the role of OPG in the pathogenesis of some co-morbidity during atherosclerotic events.[12]

Some studies have confirmed the involvement of the OPG/RANKL/RANK axis in modulating the inherent and acquired immune responses elaborated in myocardial damage. These studies have shown the involvement OPG/RANKL/RANK axis through its circulatory serum[1819,20] as well as gene expression[19] levels. However, such findings have shown contradictory results yet not solved in the clinical utility of the OPG/RANKL/RANK inflammatory pathway in UA patients. We aimed to more clarify the relationship between the serum level as well as gene expression of such pathway with severity indices of UA severity according to the thrombolysis in myocardial infarction (TIMI) and global registry of acute coronary events (GRACE) scores. If approved, it can provide a platform for explaining new studies.

MATERIALS AND METHODS

Patients and Controls

We performed this case-control study on 50 patients with UA admitted to the emergency and CCU department of Shahid Beheshti Hospital of Kashan during 2018. The Control group was composed of 50 healthy volunteers randomly selected from blood donators referring to the local blood bank. Sample size evaluated according to the other study comparing serum levels of OPG in patients suffering from acute myocardial infarction (MI) (8.04±4.86 pmol/L) and healthy controls (3.15±1.01 pmol/L).[21] G-power software calculated a sample size of at least 14 patients with a type 1 error at 5% level and 95% test power.

The inclusion criterion was considered as the patients suffering from UA. The patient's history and physical examination, electrocardiogram, and cardiac enzymes were used to diagnose the disease. We defined Non-ST Elevation acute coronary syndrome (NSTE-ACS) as the occurrence of indicative symptoms, cTnI≥0.2 ng/dL, and/or dynamic ST-segment changes (ST decrease ≥1 mm or non-persistent elevation in ≥2 contiguous derivations). UA was demarcated as the existence of expressive thoracic pain with or without repolarization abnormalities seen in the baseline electrocardiogram. Cardiac troponin I (cTnI) serum

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levels must be less than 0.2 ng/dL 24 hours after the appearance of the first symptoms. The exclusion criterion was considered for any patient with UA suffering from other inflammatory diseases at the same time. Peripheral venous blood samples were collected from all participants. The study protocol was adapted to the 1975 Helsinki ethical Declaration guidelines. The ethical committee of Kashan University of medical sciences officially approved the study (No IR.KAUMS.REC.1395.143). All participants contracted the written informed consent.

Evaluation of the Severity of UA

Evaluating the severity of UA, we used TIMI score[22,23] (the most known and most reliable system for predicting the severity of UA) and GRACE Score[23] (similar to TIMI score used for patients with non-ST elevation acute coronary syndrome (NSTE-ACS) or UA diagnosis).TIMI risk scores were calculated for every patient. TIMI score predicts the short-term risk of acute MI/coronary revascularization or death for any reason. This score assigns each of seven predictors one-point value and then classes patients into one of eight prognostic categories (Table1).

GRACE score calculates a patient's risk and guides management decisions. The GRACE score is derived from eight parts that are obtainable in primary stages (Table 2).

Table 1. Calculating the thrombolysis in myocardial infarction (TIMI) score to predict the risk of acute coronary syndrome

TIMI Risk Score for UA/NSTEMI Calculation of Risk score			
Characteristic	Point	Score	D/MI/UR by 14 d
Historical		0-1	5%
Age≥65 y	1	2	8%
≥3 risk factors for CAD	1	3	13%
Known CAD (stenosis>=50)	1	4	20%
ASA use in past 7 d	1	5	26%
Presentation		6-7	41%
Severe angina (≥episodes w/in 24 h)	1	Higher risk Pts (TRS≥3) derive ↑	
ST deviation≥0.5 mm	1	benefit from LMWH, GP llb/llla inhibitors, and	
+ cardiac marker (troponin, CK-MB)	1	early angiography	
RISK SCORE=TOTAL POINTS	0-7		

Table 2. Calculating the Global Registry of Acute Coronary Events (GRACE) score to manage the acute coronary syndrome

GRACE Calculator									
Age		**Heart Rate**		**Systolic Blood Pressure**		**Creatinine (mg/dL)**		**Killip class (assessment of cardiogenic shock)**	
Categories	**points**	**categories**	**points**	**Categorical**	**points**	**categories**	**points**	**Categories**	**points**
<40	0	<70	0	<80	63	0.0-0.39	2	Class I	0
40-49	18	70-89	7	80-99	58	0.4-0.79	5	Class II	21
50-59	36	90-109	13	100-119	47	0.8-1.19	8	Class III	43
60-69	55	110-149	23	120-139	37	1.2-1.59	11	Class IV	64
70-79	73	150-199	36	140-159	26	1.6-1.99	14	Cardiac arrest at admission	43
≥80	91	≥200	46	160-199	11	0.2-3.99	23	Elevated cardiac markers	15
-	-	-	-	≥200	0	≥4	31	ST-seg deviation	30

Experiments

Blood samples were centrifuged at 3500 rpm for 10 min and the serum was separated. Each sample was stored at –80°C. Serum levels of OPG, RANKL, and RANK were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, USA) according to the manufacturer's instructions. Total RNA was extracted from PBMC (High Pure RNA Isolation Kit, Cat No: 11828665001, Roche Applied Science). Extracted RNA was used as a template to synthesize cDNA (Transcriptor First Strand cDNA Synthesis Kit, Cat No: 04897030001, Roche Applied Science). Using the Taqman primer-probe comparative CT method, we measured the amount of OPG, RANKL, and RANK gene expression (ABI 7300 Real-time polymerase chain reaction system) according to previously published protocol.[24] Beta-actin housekeeping gene was considered as the endogenous control.

Statistical Analysis

Mean±SD and frequency/percent were calculated for quantitative and qualitative variables, respectively. We compared the groups by independent T-test as well as paired T-test in the case of quantitative factors, and by chi-square test regarding qualitative factors. The data were classified through a discriminate function model. Then, sensitivity, specificity, positive as well as negative predictive values, and CC were calculated for each model. The goodness of fit criteria for each model including canonical correlation and WILLK's lambda was calculated. Data were analyzed by SPSS19 software. A $p<0.05$ was considered as the limit of statistically significant.

RESULTS

Patients Characteristics, Serum Level, and Gene Expression of OPG, RANK, and RANKL

The demographic, clinical, and experimental characteristics of the patients with UA and healthy controls are summarized in Table 3. There were not any significant differences according to age and sex. Mean and standard deviation of serum level of RANKL in UA and healthy groups were 2.29±1.14 and 3.79±2.46 pg/L, respectively. Such values for OPG were 435.44±186.7 and 613.87±220.4 pg/L. There was a significant increase in serum levels of both OPG and RANKL in the patients compared to those in the control group ($p<0.001$) (Figures 1, 2, and 3). The mean and standard deviation of the gene expression level of RANKL in the UA group and healthy groups was 7.54±1.88 and 9.45±2.1 pg/L, respectively. Such values for OPG were 9.4±2.9 and 11.14±2.86 pg/L. The gene expression level of both OPG and RANKL were significantly higher in the patients than those in the healthy group ($p<0.001$) (Figure 4). There was no difference in serum level as well as gene expression of RANK between the two groups ($p=0.435$ and $p=0.435$, respectively) (Table 3).

Table 3. Patient's characteristics and serum level and gene expression of Osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK), and receptor activator of nuclear factor-kappa B ligand (RANKL)

Variables		Healthy group	UA group	difference	*p*
Age(Mean ± SD)		60.66±7.75	59.7±14.1		0.96
Sex	Male	19(38)	19(38)		N.S
	Female	31(62)	31(62)		
Diabetes (yes/NO)		-------	25/25		------
Grace (mean ±SD)			98.66±27.34		-----
TIMI (Mean ± SD)			2.84±0.186		-----
Serum levels	RANK (pg/mL)	93.18±38.69	97.88±45.34		0.578
	RANKL(pg/mL)	2.29±1.14	3.79±2.46		**<0.001**
	OPG (p g/mL)	435.44±186.7	613.87±220.4		**<0.001**
Gene Expression	RANK	7.14±2.42	7.32±2.24		
	RANKL	7.54±1.88	9.54±2.1		
	OPG	9.4±2.9	11.14±2.86		

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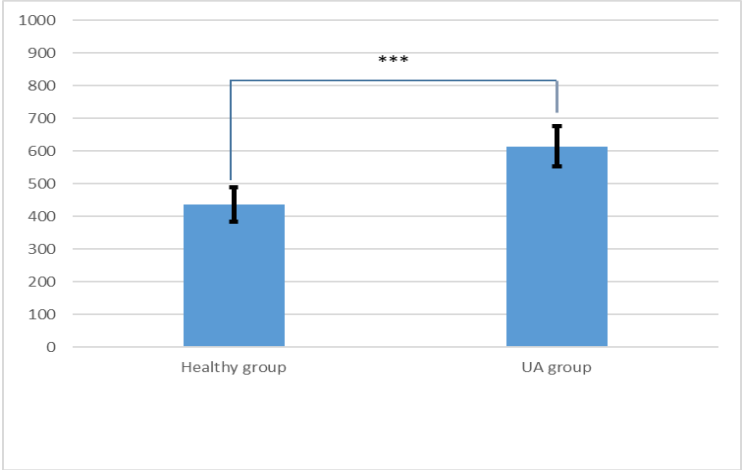

Figure 1. Mean and 95% confidence level of serum levels of Osteoprotegerin (OPG) in unstable angina patients and healthy controls. The sample size in each group is equal to 50 people. The statistical test is the Independent t-test and there is a statistically significant difference between the groups (*p<0.001).**

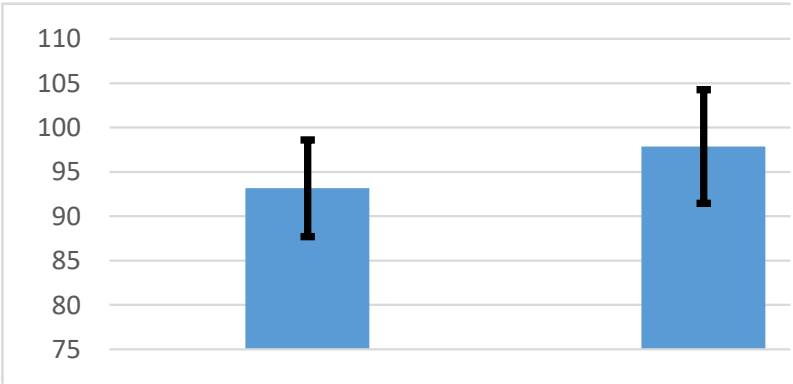

Figure 2. Mean and 95% confidence level of serum levels of receptor activator of nuclear factor-kappa B (RANK) in unstable angina patients and healthy controls. The sample size in each group is equal to 50 people. The statistical test is the Independent t-test and there is not a statistically significant difference between the groups (p=0.578).

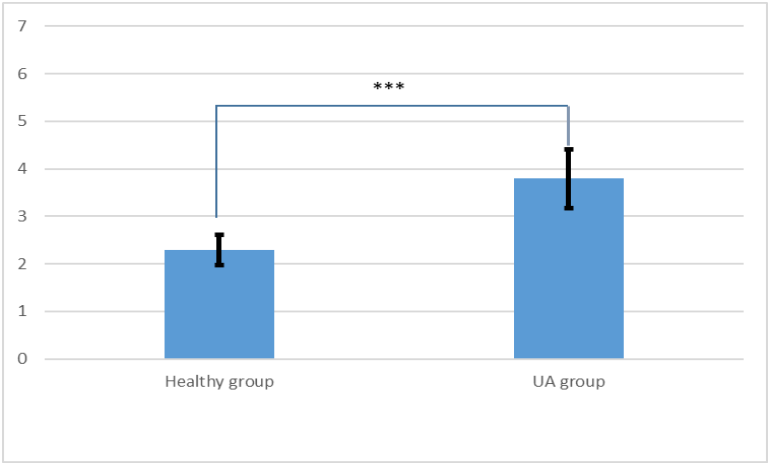

Figure 3. Mean and 95% confidence level of serum levels of receptor activator of nuclear factor-kappa B ligand (RANKL) in unstable angina patients and healthy controls. The statistical test is the "Independent t-test" and there is a statistically significant difference between the groups *** p<0.001.

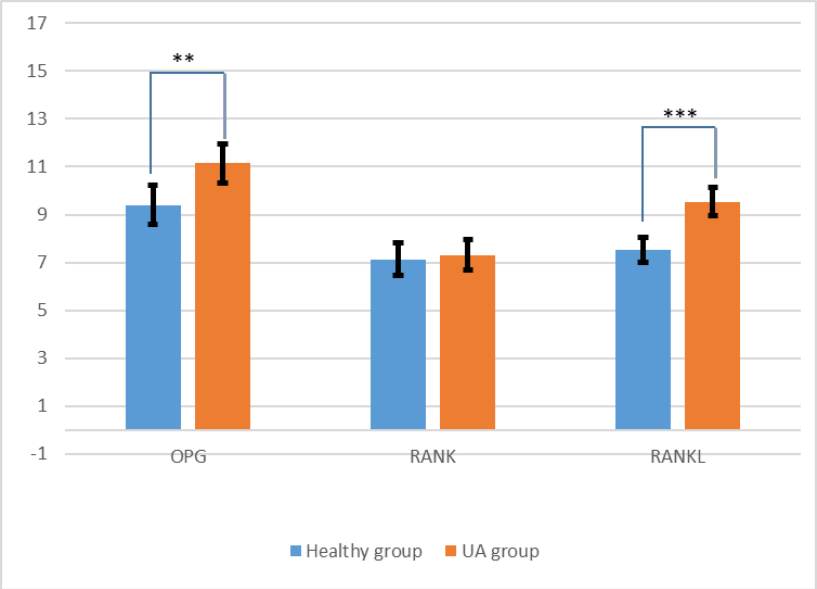

Figure 4. Gene expression levels of osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK), and receptor activator of nuclear factor-kappa B ligand (RANKL) in 50 unstable angina patients and 50 healthy controls. The gene expression level of both OPG and RANKL were significantly higher in the patients with unstable angina than those in the healthy group (p<0.001). There was no difference in gene expression levels of RANK between the two groups (p=0.435). ** p<0.01, *** p<0.001

Association of the Serum Levels and Gene Expression of OPG, RANK, and RANKL with the Severity of UA in TIMI and GRACE scores

The serum level, as well as gene expression of OPG, RANK, and RANKL in different severities, is shown in Table 4. There were no significant differences in serum levels as well as gene expression of OPG, RANK, and RANKL between low and high-risk groups according to TIMI score. Moreover, the serum levels of OPG, RANK, RANKL, and their gene expression were not significantly different between low, medium, and high-risk groups according to the GRACE score (Table 4).

Table 4. Association of the Serum Levels as well as gene expression of osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK), and receptor activator of nuclear factor-kappa B ligand (RANKL) with the severity of unstable angina (UA) in thrombolysis in myocardial infarction (TIMI) and Global Registry of Acute Coronary Events (GRACE) scores

		OPG		RANK		RANKL	
		serum	gene	Serum	gene	serum	gene
TIMI score	Low	576.4±164.2	10.3±2.3	95.9±52.4	6.8±2.4	3.9±2.3	9.5±1.9
	High	638.8±250.6	11.6±3.05	99.1±40.8	7.6±2.02	3.6±2.5	9.5±2.2
	p	0.293	0.111	0.806	0.182	0.661	0.978
Grace score	Low	631.3±209.3	10.7±2.8	95.02±48.1	7.2±2.4	3.5±2.5	9.9±2.2
	Medium	589.6±238.04	11.6±2.8	101.8±41.9	7.4±1.9	4.1±2.4	9.05±1.7
	p	0.515	0.318	0.606	0.773	0.351	0.161

Discriminant Function Parameter and the Effect of the Combination of Gene Expression of RANK, RANKL, and OPG Factors on the Prediction of UA

The results of the use of OPG, RANK, and RANKL gene expression criteria in the discriminate function analysis represented seven models, three of them were one-predictor, three of them were bi-predictors and one of them was a three-predictor model. The results showed that in models with one-predictor, the best model was RANKL, which its canonical correlation was 0.452 and its sensitivity and specificity were 62% and 70%, respectively. By adding OPG to this model (model 5), the canonical correlation increased to 0.514; and by adding RANK (full model), the canonical correlation increased to such a little value of 0.22 (0.536). The highest canonical correlation and sensitivity reached 0.536 and 76%, respectively in model 7. Removing the RANK from this model (model 5), we gained a bit increased specificity (78%) and a bit decreased canonical correlation and sensitivity (0.514 and 72%, respectively) (Table 5).

Discriminant Function Parameter and the Effect of Combination of Serum Level of RANK, RANKL, and OPG Factor on Prediction of UA

Using the OPG, RANK, and RANKL serum levels in the discriminant function analysis, we gained seven models. In one-predictor models, the best one was OPG (model 3) which its canonical correlation was 0.404 and its sensitivity and specificity were 66 % and 66%, respectively. By adding RANKL to this model (model 5), the canonical correlation increased to 0.525, while adding RANK, as a weak variable, to model 3 (model 4) the canonical correlation decreased to 0.413. The highest canonical correlation, sensitivity, and specificity were related to model 7, which is equal to 0.544, 68%, and 84%, respectively (Table 6).

Table 5. Discriminant function parameter and the effect of the combination of gene expression of receptor activator of nuclear factor-kappa B (RANK), receptor activator of nuclear factor-kappa B ligand (RANKL), and osteoprotegerin (OPG) factors on prediction unstable angina (UA)

Model	Variables	Coefficients	cc.[1]	Pred.[2]	GROUP		Sen.[3]	Sp.[4]	Ppv.[5]	Npv.[6]	cc.[7]
					case	control					
1	constant	-3.106	0.039	case	23	18	46	64	0.56	0.542	0.55
	RANK-GE	0.430		control	27	32					
2	constant	-4.287	0.452	case	31	15	62	70	0.673	0.648	0.66
	RANKL-GE	0.502		control	19	35					
3	constant	-3.568	0.292	case	26	20	52	60	0.565	0.555	0.56
	OPG-GE	0.347		control	24	30					
4	constant	-3.989	0.295	case	32	22	64	56	0.592	0.608	0.6
	OPG-GE	0.345		control	18	28					
	RANK-GE	0.062									
5	constant	-5.593	0.514	case	36	11	72	78	0.765	0.735	0.75
	OPG-GE	0.185		control	14	39					
	RANKL-GE	0.432									
6	constant	-5.681	0.479	case	33	12	66	76	0.733	0.69	0.71
	RANK-GE	0.166		control	17	38					
	RANKL-GE	0.525									
7	constant	-6.835	0.536	case	38	12	76	76	0.76	0.76	0.76
	OPG-GE	0.178									
	RANK-GE	0.149		control	12	38					
	RANKL-GE	0.460									

[1] Canonical Correlation, [2] Predicted Group Membership, [3] Sensitivity, [4] Specificity, [5] Positive Predictive Value, [6] Negative Predictive Value, [7] Corrected Classification

Table 6: discriminant function parameter and the effect of the combination of serum level of receptor activator of nuclear factor-kappa B (RANK), receptor activator of nuclear factor-kappa B ligand (RANKL), and osteoprotegerin (OPG) factor on unstable angina (UA)

Model	Variables	Coefficients	C.C[1]	Pred.[2]	Original		Sen.3	Sp.[4]	Ppv.[5]	Npv.[6]	cc.[7]
					case	control					
1	constant	-2.266		case	20	16	0.4	0.68	0.55	0.53	0.54
	RANK	0.24	0.056	control	30	34					
2	constant	-1.583		case	24	11	0.48	0.78	0.685	0.6	0.63
	RANKL	0.521	0.368	control	26	39					
3	constant	-2.568		case	33	17	0.66	0.66	0.66	0.66	0.66
	OPG	0.005	0.404	control	17	33					
4	constant	-3.080		case	33	13	0.66	0.74	0.717	0.685	0.70
	OPG	0.005	0.413	control	17	37					
	RANK	0.005									
5	constant	-3.088		case	33	12	0.66	0.76	0.733	0.690	0.71
	OPG	0.004	0.525	control	17	38					
	RANKL	0.366									
6	constant	-2.353		case	26	11	0.52	0.78	0.702	0.619	0.65
	RANK	0.008	0.386								
	RANKL	0.526		control	24	39					
7	constant	-3.844		case	34	8	0.68	0.84	0.809	0.724	0.76
	OPG	0.004									
	RANK	0.007	0.544	control	16	42					
	RANKL	0.382									

[1] Canonical Correlation, [2] Predicted Group Membership, [3] Sensitivity, [4] Specificity, [5] Positive Predictive Value, [6] Negative Predictive Value, [7] Corrected Classification

DISCUSSION

Clarifying some inconsistencies regarding the clinical utility of the OPG/RANKL/RANK inflammatory pathway in UA patients, we showed in this study significantly higher serum levels as well as gene expression of both OPG and RANKL in UA patients compared to those in the healthy group. In line with our study, some other studies have shown similar results. For example, the increased level of OPG has been shown in the circulation of patients with UA as well as MI[18] and atherosclerosis.[19] Such increased serum levels of OPG have been considered as an inflammatory marker of different phases of cardiovascular disease.[25] Furthermore, OPG binds to RANKL and reduces its rapid clearance and in this way may let RANKL be more in serum levels of UA patients[12] as there is in our patients. Other studies have shown the increased gene expression of OPG/RANKL/RANK in atherosclerosis[19] and degenerative aortic stenosis.[12]

In this study, we also showed that there is not any relationship between the OPG/RANKL/RANK axis and the severity of UA according to TIMI and GRACE scores. Other studies have reported similar results. For example, one study demonstrated that the serum levels of both RANKL and OPG are not directly correlated to the progression of atherosclerosis; however, it could contribute to the rupture and instability of vascular plaques.[20] In this study, although serum levels of OPG were acceptable as an indicator of coronary artery disease, they were not associated with the severity and degree of coronary artery disease in patients with UA and MI.[20] However, some other studies have shown opposite findings. As an instance, one study showed that OPG

serum levels are significantly associated with both the presence and severity of coronary artery disease (CAD) in patients with type 2 diabetes mellitus.[26] The other study showed a positive correlation between serum levels of OPG and the severity of CAD.[27] Such findings indicate that other soluble receptors in the TNF receptor superfamily may play some roles in the instability of plaques.

Increased levels of OPG in our patients may result from rupture or splitting of the plaque in which the increased concentration of OPG has been evidenced.[8] In line with this phenomenon, Zhang et al showed that OPG expression in the rat aorta is increased after balloon injury.[28] Any vascular injury and consequent inflammatory process within an atherosclerotic plaque lesion may cause such an increased level of OPG that manifests at its serum levels and may predict some clinical outcomes. For example, increased levels of OPG in patients receiving dialysis predict the progression of calcified plaques in the aorta;[29,30] and higher OPG levels in patients suffering from diabetes are independently associated with coronary artery calcification and predict near cardiovascular events.[31,32] It should be noted that OPG effects may be different as atherosclerotic lesion progresses. At the beginning of such ongoing detrimental stages, OPG may be increased to compensate for vasculature damage through activating inflammatory pathways.[18,33] One mechanism of such vascular protection is that increased levels of OPG at atherosclerotic plaque lesions inhibit TRAIL (TNF-related apoptosis-inducing ligand, a potent activator of apoptosis)-induced apoptosis of vascular cells.[18,33] Along with the ongoing process of atherosclerosis and in the late stages of lesion progress, OPG may cause some kind of injury to the vessels where it is incapable to reverse the process of vascular calcification.[18,34] It should be mentioned that OPG not only modulates RANKL but also acts in some RANKL-independent manners such as chemotaxis of monocytes.[35]

In our study, there were no significant differences in serum levels as well as gene expression of OPG, RANK, and RANKL among different severity groups of UA according to TIMI and GRACE scores. This finding is in line with some other studies. For example, one study demonstrated that OPG level indicates neither the quantity nor the severity of affected coronary arteries in patients suffering from CAD.[20]

Using discriminant analysis, we showed that simultaneous measurement of OPG, RANK, and RANKL serum levels as well as both OPG and RANK gene expression levels have the most value in the prediction of UA diagnosis. However, it should be noted that such values are not as valuable as other routine criteria of UA diagnosis.

The main limitation of our study was that we did just a cross-sectional study on the correlation of the OPG/RANKL/RANK axis and the severity of UA. Such measurements should also be done after the acute phase. Furthermore, role-playing factors clarifying the mechanistic effects of such axis in the process of plaque instability in UA should be defined in future studies.

Our study shows that serum levels, as well as gene expression of OPG and RANKL in UA patients, are more than those in healthy people. However, serum and gene expression levels of the OPG/ RANKL/RANK axis are not associated with UA severity.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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