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Evaluation of Coronary Artery Calcification and Gremlin-1 Serum Level Correlation in Patients with Chronic Ischemic Heart Disease

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ABSTRACT

Background: Available evidences have shown that bone morphogenetic proteins (BMPs), particularly BMP 2 and BMP 4, are involved in vascular calcification. Gremlin 1 is one of the important endogenous inhibitors of BMPs. This extracellular antagonist of bone morphogenic proteins has a very complex and cysteine-rich chain and establishes non-colonial transmissions to the members of the family with varying degrees of dependence and prevents them from binding to the receptor, thereby inhibit their function. The main objective of this study was clinical evaluation of the correlation between Gremlin-1 serum concentration and Coronary Artery Calcification.

Methods: Eighty-four patients with coronary artery disease from cardiology ward of Razavi Hospital, Mashhad, Iran, who completed the inclusion criteria, entered in the study between November 2015 and March 2016. CT-Angiography was performed to define coronary artery calcium score and Gremlin-1 serum concentrations were measured by an ELISA kit.

Results: Eighty-one patients, with mean age of 57.19±10.18 years were included to the study. The mean serum level of Gremlin-1 was 10.92±8.46 pg/mL. There was a reverse significant correlation between Gremlin-1 serum concentration and Coronary Artery Calcification of Right coronary artery(RCA) ($P<0.05$), in contrast to total Coronary Artery Calcification score, left artery Descending (LAD), Left Main (LM) and Circumflex (CX). However, there was no evidence that age and sex risk factor, hypertension, diabetes mellitus, hyperlipidemia, positive family history, current smoking and high BMI to be associated with serum level of Gremlin-1.

Conclusion: Based on the results, Gremlin-1 serum concentration may be a suitable biomarker for predication coronary artery calcification severity. However, more researches on larger population are necessary for its validation.

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Introduction

Vascular calcification is a fatal complication of cardiovascular disease. Its incidence is increasing in last year's particularly in the elderly and patients suffering from metabolic disorders. It increases mortality rate and besides, causes physical incapacities and reduces the quality of life (1,2). Actually, calcified plaques in coronary arteries are associated with almost 1.7 folds' higher incidence of mortality comparing with non-calcified ones, independent of other risk factors, making it an important none traditional risk factor of cardiovascular disease. Even in asymptomatic subjects, calcification of coronary vessels can be observed especially among the elderly, in which 60% of 60-year-olds individuals have some degree of calcification and vasculopathy (2,3). It is an unavoidable process predominantly in the advanced phases of atherosclerosis which can consequently cause plaque rupture. Coronary Artery Calcification (CAC) is a strong indicator for subclinical atherosclerosis, and future cardiovascular events especially in people with intermediate risk. There is a relationship between an increased coronary artery calcium score (CACS) and cardiovascular disease possibility (4). It should be mentioned that CACS correlates with the risk of cardiovascular events in both asymptomatic and symptomatic patients. CAC is determined by electron beam-computed tomography (EBCT) and reported as agatston score (5). Patients with a high Agatston score (>160) have an increased risk for a major adverse cardiac event (MACE) (6). Although it does not allow for the assessment of soft non-calcified plaques, it has shown a good correlation with contrast-enhanced CT coronary angiography (7). Recent studies proposed that we should move from cellular interaction based calcification models to models concentrating on the role of extracellular matrix (8). Existing data have revealed that bone morphogenetic proteins (BMPs) play an important role in vascular calcification (9). BMPs signaling pathway is too much influential in the bone formation process that its induction in the muscles results in the development of false bone tissue in that place (10). Among the proteins in this family, BMP 2 and BMP 4, and at a later stage BMP 5, 6, and 7 have the highest correlation with calcification caused vascular disease (11). BMP 2 and 7 participate in the ossification through SMAD signaling pathway. There are lots of inducers and inhibitors of BNPs; Gremlin 1 is one of the important inhibitors (12). It generates a dimmer with connection to BMP 2 and 7 and prevents them from binding to the receiver and inhibits this powerful signaling path (13,14).

This extracellular antagonist of BMPs has a very complex and rich cysteine chain and establish non-colonial transmissions to the members of the family with varying degrees of dependence, and prevents them from binding to the receptor, thereby inhibits their function (15,16). So, it seems that the elevated level of gremlin 1 in serum may be an indicator of lower risk of coronary artery calcification. According to this, we evaluated the Gremlin-1 serum level as a diagnostic biomarker in patients with chronic ischemic heart disease and its correlation with coronary artery calcium score.

Methods

Eighty-three patients with diagnosis of coronary artery disease were enrolled in this study between November 2015 and March 2016. Patients were recruited from cardiology ward of Razavi Hospital, Mashhad, Iran. All patients who fulfilled the inclusion criteria of the study and referred to the cardiologists participating in this study during the follow-up period were included. This study was accepted by ethics committee of Mashhad University of Medical Sciences (code: 931459). Patients with calcium and phosphor metabolic disorder, parathyroid disease, renal dysfunction, history of osteoarticular disorders, and zero calcium score were excluded from the study. A questionnaire containing demographic and laboratory data, medications, medical, and familial history was completed for all patients. All patients signed the consent form prior to entry in the study.

Determination of Gremlin-1 serum concentration and CAC

Whole blood was collected from patients and centrifuged at 2500 rpm for 10 min. The plasma fraction was isolated and stored at -70 °C until required for analysis. Routine biochemical measurements such as plasma glucose, total cholesterol (TC), triglycerides, low density lipoprotein Cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and serum calcium and phosphorus levels were carried out by routine laboratory methods. Serum level of soluble Gremlin-1 was measured with an enzyme-linked Immunosorbent assay (ELISA) –kit (Zellbio, Germany). Each assay was calibrated using Gremlin-1 standard curve following the manufacturer instructions. Coronary Artery Calcification score was determined by CT-Angiography and reported as agatston score. Agatston score is a semi-automated tool to calculate a score based on the extent of coronary artery calcification detected by an unenhanced low-dose CT scan. Its calculation is based on the weighted density score given to the highest attenuation value (HU) multiplied by the area of the calcification speck (Density factor: 130-199 HU: 1, 200-299 HU: 2, 300-399 HU: 3, 400+ HU: 4). For example, if a calcified speck has a maximum attenuation value of 400 HU and occupies 8 sq. mm area, then its calcium score will be 32. The score of every calcified speck is summed up to give the total calcium score. Patients were classified based on their Coronary Artery Calcification levels according to the American heart association (AHA) classification into 4 groups with zero calcium, low cardiovascular risk (calcium score 1 to 99), moderate risk (calcium score 100-400), and high risk – (calcium score was higher than 400).

The correlation of Gremlin-1 serum level and CACS in various coronary arteries was our primary outcome.

Correlation of Gremlin-1 serum level with conventional cardiovascular risk factors was also defined as secondary outcomes.

As, no previous study had evaluated the correlation of Gremlin-1 serum level and CACS we proposed this research as a pilot study and we included all eligible patients during the five-month study period. Statistical analysis was carried out by SPSS 16. All measured values are presented as mean ± SD. Correlation between serum concentrations of Gremlin-1 with CAC was analyzed using spearman correlation test, as the distribution of Gremlin-1 serum level was not normal based on Kolmogorov–Smirnov test. To compare serum concentration of Gremlin-1 between different groups, Independent-sample T Test was used. Results were considered significant at $p<0.05$.

Results

The study population consists of 83 patients, 54 males (65%) and 29 females (35%). The mean age of population was 57.19 ± 10.18 years. Patients' characteristics, laboratory tests including biochemical parameters, and traditional cardiovascular risk factors, and mean Gremlin-1 serum level and Total, RCA, LAD, LM and CX calcium score are summarized in Table 1 and 2, respectively.

The coronary arteries calcification scores distribution in various coronary arteries is summarized in Table 3.

Table 1. Demographic data, laboratory tests, and traditional cardiovascular risk factors of patients.

Demographic data	**Mean ±SD**
Age (year)	57.19 ± 10.18[1] (37-89)[2]
BMI (kg/m^2)	28.21 ± 4.69 (19.48-40.37)
Female/male ratio	29/54[3]
Laboratory tests	**Mean ±SD**
HDL-C (mg/dl)	43.00 ± 12.96 (25-121)
LDL-C (mg/dl)	92.29 ± 31.49 (42-191)
Total cholesterol (mg/dl)	164.89 ± 35.93 (109-292)
FBS (mg/dl)	105.27 ± 27.95 (75-257)
TG	142.94 ± 53.85 (17-295)
Traditional risk factors	**Frequency (%)**
Hypertension (%)	48
Dyslipidemia (%)	62
Positive family history (%)	52
Diabetes (%)	19
Current Smoking (%)	34

BMI: Body Mass Index, HDL-C: High Density Lipoprotein-Cholesterol, LDL-C: Low Density Lipoprotein-Cholesterol, FBS: Fast Blood Sugar, TG: Triglyceride . 1-Mean ± SD , 2-Minimum and maximum of range, 3-Frequency percent

Table 2. Serum concentrations of Gremlin-1 and Total, RCA, LAD, LM and CX calcium score.

Coronary artery Calcium score	**Mean ±SD**
Total calcification of coronary vessels (agatston score)	346.63 ± 557.78[1] (0-3756)[1]
Calcification in coronary LAD (agatston score)	174.73 ± 287.12 (0-1610.20)
Calcification in coronary RCA (agatston score)	65.17 ± 105.55 (0-476.80)
Calcification in coronary CX (agatston score)	45.88 ± 95.10 (0-615.20)
Calcification in coronary LMCA (agatston score)	28.20 ± 103.88 (0-749.80)
Gremlin-1 serum level	**Mean ±SD**
Concentraton of *Gremlin-1* (pg/mL)	10.92±8.46 (2.43-32)

LAD: Left Anterior Descending, RCA: Right Coronary Artery, CX: Circumflex, LMCA: Left Main Coronary Artery. 1-Mean ± SD,2- Minimum and maximum of range

Table 3. Distribution of different coronary arteries' calcification scores categories in study population.

Calcium score classification based on AHA [1]	Left anterior descending	Left main coronary artery	Right coronary artery	Circumflex
0	14.3%	55.6%	33.9%	30.2%
1-99	53.2%	36.5%	40.3%	52.3%
100-400	19.7%	6.4%	22.6%	15.9%
>400	12.8%	1.6%	3.2%	1.6%

There was a reversed significant correlation between Gremlin-1 serum level and CAC score RCA (P<0.05) but, there was no significant correlation between Gremlin-1 serum level and total coronary calcification and CAC score of LMCA, LAD and CX. (p>0.05) (Table 4).

There was no evidence that age and sex risk factor, hypertension, diabetes mellitus, hyperlipidemia, positive family history, current smoking and high BMI to be associated with serum level of gremlin1 in the multivariate analysis (Table 5).

Table 4. Correlation between Gremlin-1 serum concentration with LAD, RCA, LMCA, and CX Coronary Artery Calcification score.

Coronary Artery Calcification	P value[1]	r_s Correlation coefficient
Total CAC	0.976	0.003
CAC of LAD	0.933	0.012
CAC of RCA	<0.005*	-0.504
CAC of LMCA	0.343	-0.138
CAC of CX	0.687	-0.059

CAC: Coronary Artery Calcification, LAD: Left Anterior Descending, RCA: Right Coronary Artery LMCA: Left Main Coronary Artery, CX: Circumflex

Table 5. Multivariate logistic regression analysis of various conventional cardiovascular risk factors with gremlin-1 serum concentration.

Predictor	B	S.E.	Sig.[2]	Exp (B)	95% CI for Exp (B)	
					Lower	Upper
Age and sex risk factor[1]	0.650	0.340	0.056	1.916	0.985	3.729
BMI ≥ 30 (kg/ m²)	0.531	0.386	0.169	1.700	0.797	3.626
Diabetes mellitus	- 0.618	0.345	0.073	0.539	0.274	1.060
hypertension	0.899	0.469	0.055	2.457	0.981	6.156
positive family history	- 0.048	0.503	0.924	0.953	0.356	2.553
current smoking	0.053	0.362	0.884	1.054	0.519	2.142
dyslipidemia	0.780 -	1.174	0.506	0.459	4.575	0.046

[1] Age above 55 for women and 45 for men

[2] Multivariate logistic regression

Discussion

It was the first human study for evaluation of the correlation between Gremlin 1 serum level and CAC. There was a reversed significant correlation between Gremlin-1 serum level and RCA CAC score (P<0.05), but there was no significant correlation between Gremlin-1 serum level and total coronary calcification and CAC score of LMCA, LAD and CX. (p>0.05).

Just a human study is performed by Munoz et al., at 2017, which evaluated Gremlin 1 serum level relation with the presence of subclinical atherosclerosis, assessed by measures of CAC in patients without known coronary artery disease (CAD). They found a Gremlin 1 deficiency in CAC+ patients without known CAD (17).

In a study by Li-feng et al., on 40 patients with end stage renal disease, the relationship between Gremlin 1, BMP2 and BMP 7 expression and degree of radial arteries calcification was investigated. In this study was observed that there is a significant positive relationship between Gremlin-1 expression and vascular calcification, exactly in contrast to BMP 7 (18). These findings are completely in contrast with theoretical concepts. However, in current study, we excluded patients with renal impairment and the present study is the only clinical study that investigated the relationship between serum levels of Gremlin-1 and coronary calcification in patients with chronic ischemic heart disease. In another study conducted by Jara et al., considering the inhibitory effect of BMP-7 on vascular calcification and the BMP-2 collaborative effect in this

process, Gremlin-1 was studied as antagonist of BMP-2 and 7. In this study, which performed on uremic rats, Gremlin- 1 was evaluated by immunohistochemistry and vascular calcification by Von Kossa Staining method and a significant negative correlation between Gremlin- 1 level and arterial calcification was observed. In addition, in this study, 16 biopsied veins of uremic kidney patients were studied and the same result was obtained. It was also revealed that increase in gremlin level increases the migration and proliferation of Vascular Smooth Muscle Cells (VSMCs) and its expression significantly increases in vascular damage. They observed a marked gremlin overexpression in the media layer of vessels in uremic rats and patients in association with vascular calcification and BMP-2 expression. They proposed that gremlin may play a role in the vascular calcification process in uremia, and its interaction with BMP-7 or BMP-2 remains to be elucidated. Gremlin could mediate its action via induction of epithelial to mesenchymal feedback signaling, as it has been suggested that gremlin has a role in the EMT of tubular cells in renal tubule interstitial fibrosis, and in the glomerular crescent formation. Whether this overexpression at the medial vascular layer from uremic patients and azotemic rats with calcitriol-induced vascular calcification is contributing to enhance mineral deposition blocking the beneficial action of BMP-7 or might have a salutary effect by blocking actions of BMP-2-cascade signaling of calcification remains to be established (19).

In several studies, inhibitory effects of Gremlin-1 on BMP 2 in the vascular calcification process have been assessed (20). For example, in a study performed by Muller et al., serum level of Gremlin-1 was measured by ELISA kit in 120 patients with ACS, 166 patients with CAD and 25 control subjects. Its serum levels were significantly higher in ACS compared with stable CAD and healthy control subjects. They considered the ratio of Gremlin-1 to macrophage migration inhibitory factor (MIF) and it was independently correlated with the incidence of ACS and intracoronary thrombus burden. Actually it indicated instability of CAD (21). Moreover, the correlation of BMP4 (which could be antagonized by Gremlin-1) and CVD was assessed. Park et al. reported that serum level of BMP4 was significantly lower in patients with multivessel disease (MVD) in comparison with single vessel ones. A high serum BMP4 level was an independent predictor for a decreased risk of MVD (22).

According to the studies on the Gremlin-1 bioassay which have been carried out in cell culture or animal experiments, similar results have been reported in limited number of human studies which are carried out on this subject. This significant negative correlation was also found by CAC of RCA, but not the other coronary arteries. It may be due to non-uniform distribution of other vessels' calcium scores resulting from small sample size. If a larger sample size was evaluated, a significant correlation between total calcification of coronary arteries and serum levels of Gremlin- 1 may be found. According to statistical results, indicated a significant negative correlation between serum levels of Gremlin- 1 and RCA calcification, and no relationship was found between serum concentrations of this biochemical index and other vessels.

In this study, the correlation of the Gremlin-1 serum level with CAC was clinically evaluated for the first time in patients with coronary artery disease that there was a reversed significant correlation between Gremlin-1 serum level and CAC of RCA ($P<0.05$), but there was no a significant correlation between gremlin-1 serum level and total CAC and CAC of LAD, LM, CX ($P>0.05$).

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