Up-regulated IncRNA-PVT1 Expression in Peripheral Blood Mononuclear Cells of Patients with Coronary Artery Disease is Correlated with Decreased Interleukin-10 Production

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Abstract

Background: Plasmacytoma variant translocation 1 (PVT1) is a newly discovered long non-coding RNA (lncRNA), and it has not been previously studied in the inflammatory responses of peripheral blood mononuclear cells (PBMCs) of patients with coronary artery disease (CAD).

Methods: This cross-sectional study was conducted in 15 CAD patients and 15 non-CAD (NCAD) individuals. PVT1 expression in PBMCs of the participants was measured, using real-time PCR.

Interleukin (IL)-10, IL-22 and MMP-9 in the plasma and supernatant of the cultured PBMCs in the presence or absence of lipopolysaccharide (LPS) was assessed, using flowcytometry and ELISA.

Results: An increased expression of PVT1 was observed in untreated PBMCs of CAD patients compared to the NCAD group. There was a significant up-regulation of PVT1 after LPS treatment in PBMCs of both groups. Plasma matrix metalloproteinase-9 (MMP-9) levels were found to be higher in CAD patients compared to the controls. The level of IL-10 and IL-22 production from the non-treated PBMCs of CAD was significantly lower compared to the NCAD group. In the total examined population, PVT1 expression was negatively correlated with IL-10 secretion. The results also showed a significant negative correlation between PVT1 expression and IL-10 produced by untreated cells.

Conclusions: PVT1 expression is increased in PBMCs of CAD patients and this increased expression could be associated with decreased IL-10 production from PBMCs of these patients.

Background:

Coronary artery disease (CAD) is considered as principal cause of death and disability leading to increased health economic burden worldwide (1). The most common form of CAD is atherosclerosis which is a chronic inflammatory disease of the arterial wall, arising from a maladaptive inflammatory response and an imbalance in lipid metabolism (2). Numerous converging lines of clinical and experimental evidence show that inflammation plays a pivotal role in all phases of the atherosclerotic process (3).

Previously, the role of inflammatory cytokines has been remarkably evaluated (4). It has been shown that pro-inflammatory cytokines, e.g. interleukin (IL)-1 β , and IL-6, and tumor necrosis factor alpha (TNF- α) present a pro-atherogenic effects in general, while anti-inflammatory cytokines, e.g. IL-10, could reduce the risk of atherosclerosis progression and ameliorate the development of atherosclerosis and vascular complications (5). IL-22, a member of the IL-10 related cytokine superfamily, has a dual nature in inflammation, which might present a pro-inflammatory or anti-inflammatory effect in order to modulate the tissue's immune responses (4). All in all, it seems that IL-10 and IL-22 have beneficial effects in protecting against metabolic disorders, decreasing chronic inflammation and related complications (6).

Matrix metalloproteinases (MMPs) play a crucial role in extracellular matrix metabolism. MMPs have shown the important involvement in cardiovascular diseases, including atherosclerosis, coronary artery disease, and myocardial infarction (7). MMP-9 plays a pivotal role in the instability of atherosclerotic plaques. It can also be found in the plasma due to the proteolytic rupture of the cellular membranes. Therefore, MMP-9 can be evaluated in several clinical situations in which inflammation is an underlying contributor, e.g. CAD (8).

Long non-coding RNAs (lncRNAs), a new class of non-coding RNAs with a length of > 200 nucleotides, are considered as a significant component in epigenetic regulation of gene expression. Increasing evidence has demonstrated that lncRNAs are involved in the regulation of numerous physiologic and pathologic processes, particularly the regulation of injury, invasion and inflammatory responses (9).

Plasmacytoma variant translocation 1 (PVT1) is a newly discovered lncRNA and is located at chromosome 8q24, a known cancer-related region. PVT1 functions as an oncogene and its up-regulation is tightly associated with a series of human cancer types (10). PVT1 dysregulation is involved in the etiology of a wide variety of human disorders such as cardiovascular diseases (11). During the past few years, PVT1 has received increasing interest due to its involvement in the aberrant inflammatory and immune responses in various diseases, including osteoarthritis (12, 13), epilepsy (14), abdominal aortic aneurysm (15), and sepsis-induced acute kidney injury (16) and cardiac dysfunction (17). Thus, previous literature has highlighted the potential of PVT1 as a promising therapeutic target for inflammatory disorders. However, the importance of PVT1 on inflammatory responses in CAD and atherogenesis has not been thoroughly elucidated to date. Therefore, to investigate the possible role of PVT1 in immune and inflammatory responses in CAD, in the present study, we established an experimental model of lipopolysaccharide (LPS) stimulated human Peripheral blood mononuclear cells (PBMCs). Using our model, we detected the expression of PVT1 in PBMCs from patients with CAD under both LPS-stimulated and unstimulated conditions. Furthermore, the correlation of PVT1 expression with plasma levels and the *ex-vivo* production of IL-10 superfamily cytokines (IL-10 and IL-22), and MMP-9 by PBMCs was investigated.

Methods

Study design and participants

This study was approved by medical ethics committee of Fasa University of Medical Sciences and an informed written consent was obtained from all the participants. Our study was performed in accordance with the principles of the Declaration of Helsinki (18). Thirty peripheral blood samples were collected from male patients who had undergone CT angiography or coronary angiography for suspected coronary artery disease in the Al Zahra Heart Hospital (Shiraz, Iran) between October 2018 and January 2019. We divided the patients into two groups including CAD and non-CAD (NCAD), each containing 15 patients. The diagnosis of CAD was made by a cardiologist if any patients had more than 50% stenosis in at least one coronary artery proven by angiography. Exclusion criteria were diabetes mellitus, malignancy,

infection, blood diseases and chronic renal or liver failure, history of inflammatory diseases, and use of immunosuppressive drugs.

Isolation and culture of PBMCs

To prepare the PBMCs, 10 ml peripheral blood samples drawn from participants were collected in sterile tubes with EDTA. Centrifugation was done over Ficoll-Hypaque gradients (Lymphodex, InnoTrain, Germany) at 400g for 20 min at 4°C. The interface fraction containing PBMCs was carefully collected and washed twice with RPMI 1640 medium. The PBMCs were cultured in the RPMI medium supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum at 37°C in 5% CO₂ incubator. In order to evaluate the PVT1 expression, 2×10^6 PBMCs/well were incubated in the presence or absence of 100 ng/mL of LPS (Sigma-Aldrich, Germany) for 5 hours in 24-well culture plates. Also, 10^5 PBMCs/well were cultured in the presence or absence of 100 ng/mL LPS with 48 hours of incubation in 96-well culture plates in order to evaluate the amounts of IL-10, IL-22, and MMP-9 in cell culture supernatants

RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR)

The total RNA from PBMCs cell cultures was extracted using the One Step-RNA Reagent (Bio Basic, Germany), according to its manufacturer's protocol. First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The qRT-PCR for PVT1 beta-actin as the internal reference gene was carried out, using the ABI 7500 Sequence Detection System (Applied Biosystems, USA). The primer sequences were as follows: PVT1: Sense 5'-GTCTTGGTGCTCTGTGTT-3', anti-sense 5'-CCCGTTATTCTGTCCTTCTC-3'; Beta-actin: Sense 5'-GCCTTTGCCGATCCGC-3', anti-sense 5'-GCCGTAGCCGTTGTCTG-3'. The $2^{-\Delta CT}$ method was used to calculate the relative gene expression.

MMP-9 measurements

MMP-9 in the plasma and PBMCs cell culture supernatants was detected, using MMP-9 ELISA kit from BT LAB (BT LAB, China) according to the manufacturer's instructions.

Cytokines assay

The plasma samples and the supernatants of the cultured PBMCs were used to assess IL-10 and IL-22 levels. Cytokines were measured by LEGENDplex™ Human Th22 Panel (Biolegend, USA), using the flowcytometer (FACS Calibur, BD, USA), according to the manufacture's instruction.

Statistical analysis

The SPSS 22.0 (IBM Inc., USA) and GraphPad Prism version 8.2 (San Diego, CA, USA) were used to perform statistical analysis. The normality of the continuous variables was evaluated, using Kolmogorov-Smirnov test. Normal variables are presented as mean and standard deviation (SD) and non-parametric variables are presented by median and interquartile range (IQR). The study groups were compared using

Student's T test, Mann-Whitney U test or one-way ANOVA. The Spearman correlation test was used to determine the relationship of PVT1 expression with other variables. $P < 0.05$ was regarded statistically significant.

Results

Characteristics of the study population

The demographic and anthropometric characteristics of the study population are as presented in Table 1. There were no significant differences in age, waist circumference (WC), hip circumference (HC), body mass index (BMI), and weight to hip ratio (WHR) between the study groups.

Table 1. The demographic and clinical characteristics of the study groups			
Variable	NCAD (n=15)	CAD (n=15)	P value
Age (years)	56.20 (5.82)	58.87 (5.76)	0.217
WC (cm)	94.0 (89.0-97.0)	93.0 (86.0-98.0)	0.653
HC (cm)	85.67 (7.49)	80.80 (6.48)	0.067
BMI (kg/m ²)	26.60 (2.31)	25.59 (3.34)	0.344
WHR	0.91 (0.12)	0.86 (0.11)	0.240
CAD: coronary artery disease, NCAD: non-CAD, WC: waist circumference, HC: hip circumference, BMI: body mass index, WHR: weight to hip ratio.			

PVT1 expression in PBMCs

The relative expression of PVT1 were first assessed at the basal stage in PBMCs of NCAD and CAD groups. The relative expression of PVT1 was significantly higher in CAD patients compared to the NCAD group ($p = 0.034$) (Fig 1). In addition, PVT1 expression in PBMCs of NCAD ($p = 0.002$) and CAD ($p = 0.001$) increased significantly upon stimulation.

Fig 1. PVT1 expression in the PBMCs of patients with CAD and NCAD. PVT1 relative expression in PBMCs of the CAD and NCAD patients in the absence and presence of LPS (100 ng/mL) for 5 h was measured, using real-time PCR. * P value < 0.05 ; ** P value < 0.01

Plasma levels of MMP-9, IL-10 and IL-22

Plasma levels of MMP-9 and cytokines IL-10 and IL-22 were measured to determine the inflammatory conditions as well as their correlation with PVT1 expressions. As presented in Fig. 2, only MMP-9 was significantly ($P = 0.037$) higher in CAD patients compared to the controls, and the levels of cytokines were comparable between the investigated groups.

Fig 2. Comparison of MMP-9, IL-10 and IL-22 in the plasma of NCAD and CAD patients. (A) MMP-9 was significantly higher in CAD patients. (B and C) IL-10 and IL-22 was comparable between NCAD and CAD patients. * P value < 0.05

MMP-9, IL-10 and IL-22 production by PBMCs

In order to assess the effect of LPS and correlation of PVT1 with MMP-9 and anti-inflammatory cytokines production, we also analyzed the supernatant of PBMCs in the absence and presence of LPS after 48 h. The level of IL-10 production was significantly lower in the non-treated PBMCs of CAD (p=0.002) and LPS-induced PBMCs of the NCAD group (p = 0.048) compared to non-treated PBMCs of the NCAD group (Fig 3B). The secretion of IL-22 was also significantly lower in the untreated PBMCs of the CAD group compared to the NCAD group (p = 0.044) (Fig 3C).

Figure 3. Alterations in MMP-9, IL-10 and IL-22 production, following stimulation of the isolated PBMCs with LPS. Isolated PBMCs were cultured in the presence or absence of LPS (100 ng/mL) for 48 h. Then, concentrations of MMP-9, IL-10 and IL-22 were measured in the culture supernatants. * P value < 0.05 and ** P value < 0.01

Association of PVT1 with MMP-9, IL-10 and IL-22

Correlation analyses were performed to assess the association of PVT1 expression with MMP-9, IL-10 and IL-22 cytokines expression in PBMCs (Table 2). A significant negative correlation was observed between PVT1 expression and supernatant IL-10 in the supernatant of untreated PBMCs (r = -0.399, p = 0.043).

Table 2. The correlations between PVT1 expressions with other variables		
Variables	Untreated	LPS-treated
Plasma MMP-9 (pg/ml)	0.220	-0.023
Plasma IL-10 (pg/ml)	0.008	0.089
Plasma IL-22 (pg/ml)	-0.138	0.034
Supernatant MMP-9 (pg/ml)	0.307	0.186
Supernatant IL-10 (µg/ml)	-0.399*	-0.345
Supernatant IL-22 (µg/ml)	-0.361	-0.528
MMP: Matrix metalloproteinase, IL: Interleukin. The Bold value is statistically significant. * P value < 0.05		

Discussion

Our data demonstrated that PVT1 was significantly up-regulated in the PBMCs of the CAD patients. Up- or down-regulated expression of CAD has been reported from different pathological states. It was reported that PVT1 expression is decreased in the dorsal root ganglia of diabetic rats (17). In addition, Wang et al. reported that down-regulated long non-coding RNA PVT1 contributes to human gestational diabetes mellitus and preeclampsia and the PVT1 expression was lower in the gestational diabetes mellitus and preeclampsia placentas than the normal placentas (19). Another study reported that PVT1 was down-regulated in clinical villi samples from spontaneous abortion (20). In contrast to these findings, an increased expression of PVT1 has also been previously reported. The level of PVT1 expression was significantly increased in cancers. A meta-analysis on a total of 23 studies reported that high PVT1 expression levels correlated with poor overall survival, larger tumor size, distant metastasis, and some other risk factors in cancer patients (21). In addition, it is found to be up-regulated in atrial muscle tissues from atrial fibrillation patients (22), and the myocardial tissues of sepsis rats (23). More importantly, a very recent study by Quan et al. reported a similar finding to our findings, in which PVT1 was highly expressed in the serum of CAD patients (24). Furthermore, it was demonstrated that PVT1 directly correlated with Gensini score in CAD patients, suggesting the involvement of PVT1 in the development of atherosclerosis. In this paper, PVT1 was highly expressed in PBMCs of CAD patients compared to healthy controls, and also up-regulated upon LPS-stimulation in both groups.

To study the role of PVT1 in the production of the IL-10 super family cytokines and MMP-9, we assessed the IL-10, IL-22, and MMP-9 production in both basal and LPS treated states. IL-10 is a pleiotropic cytokine with immune-regulatory properties, produced mostly by Tregs and B regulatory cells. Due to its anti-inflammatory properties, IL-10 is believed to have inhibitory effects on the plaque development and atherosclerosis progression (25). In the present study, we also found that as PVT1 increases, the production of IL-10 is reduced from PBMCs of the total population examined. Therefore, PVT1 might be the factor contributing to anti-inflammatory cytokine IL-10 secretion from PBMCs.

To the best of our knowledge, this is the first to show the relationship between PVT1 expression and IL-10 production in NCAD and CAD patients. Zhao et al. reported high expression of PVT1 in the cartilage of osteoarthritis patients and IL-1 β -stimulated chondrocytes. Moreover, in support of this finding, it has been reported that a defect cessation of PVT1 inhibition antagonized the production of inflammatory cytokines upon IL-1 β stimulation, including prostaglandin E2, IL-6, IL-8 and TNF- α . However, the precise molecular mechanisms of this correlation is not well understood; however, different factors might be involved, such as anti-inflammatory microRNAs, e.g. microRNA (miR)-146a, miR-27b-3.

Several studies have reported the association between miR-14a and CAD; the results of a recent systematic review and meta-analysis supported the existence of a role for miR-146a rs2910164 polymorphism in determining the susceptibility to cardio-cerebrovascular diseases, especially CAD (26). Another study reported that the expression of miR-146a in PBMCs and plasma was significantly higher in CAD patients compared to NCAD patients (27). In addition, it has been suggested that PVT1 is a mediator of miR-146a expression by inducing the methylation of CpG Island in its promoter so that the miR-146a overexpression eliminates the effects of PVT1 knockdown on prostate cancer cells. Liu et al. showed that

miR-146a was down-regulated and negatively correlated with the PVT1 level in prostate cancer. PVT1 mediated miR-146a expression by inducing the methylation of CpG Island in its promoter. MiR-146a overexpression eliminated the effects of PVT1 knockdown on prostate cancer cells (28). It should be noted that miR-146a, as a target of PVT1, has been documented as an anti-inflammatory mediator (29), which not only correlates with increased production of anti-inflammatory cytokines TGF- β 1 and IL-10 (30), but also distinguishes CAD patients from NCAD patients, as mentioned above. MiR-27b-3p could be another possible mediator to justify the observed correlation between PVT1 and IL-10. Complementary sequences of PVT1 and miR-27b-3p has been predicted. In addition, the effect of PVT1 on miR-27b-3p has been previously evaluated in C28/I2 human chondrocyte cell line, and results showed that overexpression of PVT1 significantly decreased, and interference of PVT1 increased the miR-27b-3p expression (13). Rouas et al. showed that overexpression of miR-27b-3p, down-regulated IL-10 expression upon targeting its 3'-UTR in T-cells; however, to the best of our knowledge, the deregulation of miR-27b-3p in CAD patients has not been reported yet (31).

In this study, we also found a higher level of MMP-9 in the plasma of CAD patients compared to healthy controls. However, in spite of some other studies in other pathologic conditions, e.g. in aortic aneurysm model (12), and lung cancer cell lines (23), no significant correlation was observed between PVT1 expression and plasma or supernatant levels of MMP-9.

So far, there has been relatively little research conducted on the relationship between IL-22 and coronary artery disease. According to the study of Gong et al., the elevated serum IL-22 was associated with the incidence of type 2 diabetes mellitus and CAD. Furthermore, they showed IL-22 was able to protect the endothelial cells against lysophosphatidylcholine high and glucose-induced injury (32). In the present study, our results demonstrated that the serum IL-22 level elevated in response to the LPS-induced inflammation. Therefore, it could be suggested that keeping IL-22 levels high, through inhibiting inflammation, can be another therapeutic target for microvascular diseases with chronic low-grade inflammation, such as diabetes and CAD. However, interleukin has been introduced as a double-edged sword (32), and more studies are needed in this area.

Conclusion

PVT1 expression is increased in the PBMCs of CAD patients and this increased expression could be associated with decreased IL-10 production from PBMCs of these patients. However, further *in-vivo* and *in-vitro* studies are required to explore the precise molecular mechanism of PVT1 up-regulation and its association with inflammatory responses in CAD patients.

Abbreviations

Coronary artery disease

CAD

tumor necrosis factor alpha

TNF- α
Long non-coding RNAs
lncRNAs
Matrix metalloproteinases
MMPs
Plasmacytoma variant translocation 1
PVT1
Peripheral blood mononuclear cells
PBMCs

Declarations

Ethics approval and consent to participate: Prior to data collection, all respondents were informed about the aims of the study and data confidentiality, and gave written informed consent. All participants took part in the survey voluntarily. ethic No. IR.FUMS.REC.1397.144.

Consent for publication: not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors have declared that no conflict of interest exists (financial, non-financial) and they have consent to the publication.

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Authors' contributions: Conceptualization: BM, PNS methodology: PNS, MK, ME, data curation and analysis: BM, PNS, AS, writing—original draft preparation: PNS, writing—review and editing: BM, AS, PNS, the authors read and approved the final manuscript.

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Figures

Figure 1

PVT1 expression in the PBMCs of patients with CAD and NCAD. PVT1 relative expression in PBMCs of the CAD and NCAD patients in the absence and presence of LPS (100 ng/mL) for 5 h was measured, using real-time PCR. *P value < 0.05; **P value < 0.01

Figure 2

Comparison of MMP-9, IL-10 and IL-22 in the plasma of NCAD and CAD patients. (A) MMP-9 was significantly higher in CAD patients. (B and C) IL-10 and IL-22 was comparable between NCAD and CAD patients. * P value < 0.05

Figure 3

Alterations in MMP-9, IL-10 and IL-22 production, following stimulation of the isolated PBMCs with LPS. Isolated PBMCs were cultured in the presence or absence of LPS (100 ng/mL) for 48 h. Then, concentrations of MMP-9, IL-10 and IL-22 were measured in the culture supernatants. * P value < 0.05 and ** P value < 0.01

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