

Downregulation of AnnexinA5 Gene Expression in Coronary In-Stent Restenosis – A Pilot Study

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Research Article

Keywords: In-stent restenosis, AnnexinA5, Angioplasty, Expression

Posted Date: June 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-459120/v1>

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Abstract

Background and aims:

In-stent restenosis (ISR) is the Achilles heel of angioplasty. AnnexinA5 as an anticoagulant has been shown have anti-inflammatory and anti-atherosclerotic effects. Here we aim to investigating the mRNA expression of AnnexinA5 in peripheral white blood cell of patients with in-stent restenosis.

Methods

Patients with the history of coronary stent implantation who candidate for re-angiography were entered the study and allocated into two groups according the results of re-angiography; in-stent restenosis (stenosis \geq 50% in stent) and non-in-stent restenosis (stenosis < 50% in stent). Total RNA of WBC was extracted and cDNA was synthesized using commercial kits. AnnexinA5 expression was assessed with real time PCR and TaqMan probe and reported in relation with GAPDH as a housekeeping gene.

Result

AnnexinA5 expression was investigated in total 50 participants including 25 ISR and 25 non-ISR. Baseline characteristics including age, sex, smoking habits, hypertension, diabetes mellitus, dyslipidemia and stent in LAD were statistically the same in cases and controls. AnnexinA5 expression in ISR patients was 50% lower than controls.

Conclusion

AnnexinA5 is down-regulated in ISR and could be considered as a biomarker for predicting ISR and furthermore it could be used as prevention for ISR occurrence.

Introduction

One of the most important methods which were developed for the treatment of coronary artery atherosclerosis is coronary angioplasty. It revolutionized the field of cardiology. Coronary angioplasty reduced the need for coronary artery bypass graft (CABG) surgery for patients suffering from coronary artery disease (1, 2). Angioplasty was first performed in 1977 by Gruentzig (3).

Despite many advances in this field and the rate of initial success and low complication rate, restenosis is considered as the Achilles heel of angioplasty and is a major clinical problem (4, 5). In various studies, the rate of restenosis has been reported between 15 and 40% (6, 7). Different methods have been used to prevent restenosis. Stent placement is one of the most important ones that has reduced the rate of

restenosis but in-stent restenosis (ISR) is one of the clinical problems in interventional cardiology with a success rate of 15 to 27%(8-10).

Various risk factors for ISR have been mentioned that can be divided into three groups: Patient- related Causes such as age, sex, genetics, and underlying diseases, Lesion -related Causes such as the length of the lesion, the location of the lesion and Procedure-related Causes such as number of stents, type of stent (11-14).

Genetic backgrounds are one of the patients – related causes that are obtained by genome wide association studies (GWAS) or candidate gene study based on results of previous study or biological pathways related to restenosis (15, 16). Evidence showed that factors which are inherited, can partly define reasons for the higher risk of restenosis incidence in patients after percutaneous coronary interventions. These agents include gene polymorphisms, which are the cause of various alterations in the gene products. Numerous studies have been focused on polymorphic alleles of genes encoding proteins related to restenosis (17). One of these proteins is AnnexinA5, a member of the Annexin family that was first known as anticoagulant proteins (18). In addition to its inhibitory role in clot formation and thrombosis, its role as an anti-inflammatory and anti-atherosclerotic agent has been revealed in studies (19-21). Increasing amounts of atherosclerotic diseases and plaque vulnerability are the causes of vascular inflammation which can be adjust in order to prevent disease progression (22). AnnexinA5 which is abundant in atherosclerotic plaques binds reversibly, specifically and with high affinity to externalized membrane PS (phosphatidyl-serine) of cells those initiating apoptosis and inflammation, result in PS-mediated platelet and leukocyte adhesion prevention, it has an impact on systemic inflammation and result in better endothelial function. In addition, anxA5 also exerts its anti-inflammatory role through its relevance to IFN- γ receptor, it can inhibit cellular inflammatory responses when IFN- γ release (23). Due to the important role of inflammation in restenosis, AnnexinA5 as an anti-inflammatory can play a role in restenosis.

Based on the results of a previous study by our research team, polymorphism of Anexina5 gene (ANXA5-1247T/C) was significantly related to coronary restenosis in recessive genetic model (24). In the present study, we investigated the expression rate of anxA5 in white blood cells of two groups including in-stent restenosis (ISR) patients in comparison with the none in-stent restenosis (NISR) cases.

Materials And Methods

Participants and sample collection:

Total 50 patients who were referred for re-angiography because of ischemic coronary symptoms after previous angioplasty were enrolled to this study between December 2015 and June 2018. According to the results of the second angiography, 25 patients who had more than 50 percent stenosis in coronary stent were entered in the ISR (in-stent restenosis) group and 25 patients who had patent stent or lesser than 50 percent stenosis in coronary stent, were entered in the NISR (non in-stent restenosis) control

group. The Scientific Ethics Committee of the Medical University of Isfahan, Iran approved the study protocol (2015/930834) and all patients provided written informed consent.

Patients with chronic kidney disease (CKD), primary percutaneous coronary intervention (PCI), thrombophilia, active cancer, and autoimmune disease were excluded.

Extraction of RNA and synthesis of cDNA:

5 mL venous blood was collected in EDTA and RNase free tube from all cases for RNA extraction.

After buffy coat isolation from blood we normalized number of WBC by WBC counting using Sysmex XP-300 automated hematology analyzer and selected the sample contains approximately 10000 white blood cells for RNA extraction (The first step of normalization) .Total RNA was extracted using TriPure Isolation Reagent kit (Roche, Germany) based on the manufacturer's instructions. The concentrations and purity of isolated RNA samples were checked by Nanodrop at 260 and 280 nm. cDNA Synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen, USA) according to the manufacturer's protocols.

We used GAPDH exon junction primer to confirm the cDNA synthesis, by this PCR reaction protocol: Initiation phase 94° C for 4 min, 35 cycles amplification phase 94° C for 60 s, 60° C for 60 s and 72° C for 45s , and final extension phase at 72° C for 5 min. At last, the PCR products became visible on gel electrophoresis.

Relative quantification Real Time PCR:

The Anxa5 gene expression levels in ISR and NISR groups were measured by relative quantification real time PCR in Roche LightCycler96 instrument. We used predesigned Taqman Primer-Probe and TaqMan Universal Master Mix from Applied Bio system company (ABI, USA). The total reaction volume was 20 µl contained 10 µl TaqMan Universal Master Mix, 1µl (600ng/ µl) cDNA (the second step of normalization), 1µl (10 pmol/ µl) of AnxA5/GAPDH Taqman primer/probe and 8 µl ddH2O (Table1).

Real time PCR reaction according to ABI protocol was as following:

The first step preincubation: 50° C for 120s the second preincubation phase 95° C for 600s and 40 two-step amplification cycles: 95° C for 15 s, 60° C for 60 s. Because we used TaqMan probe there was no need to melting phase. GAPDH gene was used as internal control (the third step of normalization). All real time PCR reactions were done duplicated.

Statistical analysis:

The SPSS statistical software v22 (IBM, Chicago, IL) was used for statistical analyses. Continuous and categorical data were reported as mean ± standard deviation and percentage respectively. Kolmogorov-sminrov test used to test the normality distribution of Δ CT .Mann-Whitney test was used to compare the

expression of annexinA5 gene in the two groups of restenosis and non-restenosis, considering that the distribution of Δ CT in the two groups was not normal.

Results

Analysis for the expression of Anxa5 gene was performed in 25 in stent restenosis(ISR) cases and 25 non in stent restenosis(NISR) matched cases

Table 1: Concentration and volume of reagents in Real Time PCR reaction

Volume	Concentration	Reagent
10 μ l	5x	Taqman Universal Master Mix
1 μ l	10 pmol/ μ l	Taqman Probe/Primer Annexin5/Gapdh
1 μ l	600 ng/ μ l	CDNA
8 μ l	-	dH2O
20 μ l	-	Total Volume

Demographic data was revealed in table 2.The mean age of the case group (ISR) was 60.70 years old and control group (NISR) was61.96 years old.34 patients were male and 16 patients were female.

Table 2: Demographic characteristics of cases

Variables	NISR (N=25)	ISR (N=25)	P value
Age (y)	61.96 \pm 8.95	60.70 \pm 8.80	0.230
Sex (n (%))	Male	15 (60%)	0.182
	Female	10 (40%)	
Positive smoking habit (n(%))	5 (20%)	4 (16%)	0.135
Hypertension (n (%))	16 (64%)	14 (56%)	0.387
Diabetes mellitus (n (%))	11 (44%)	14 (56%)	0.286
Dyslipidemia (n (%))	14 (56%)	12 (48%)	0.389
Stent in LAD (n (%))	12 (48%)	18 (72%)	0.074
Systolic BP (Mean \pm SD)	125.8 \pm 16.7	122.9 \pm 12.2	0.113
Diastolic BP (Mean \pm SD)	77 .5 \pm 9.1	78.1 \pm 7.8	0.505

To optimize the qPCR assay, we performed serial dilutions of a template and used the results to generate a standard curve. Based on the standard curve result the efficacy of PCR for both genes was acceptable and GAPDH gene was suitable for normalization (table3).

Table 3: Standard curve results based on PCR of serial dilutions of a template

Standard curve criteria	AnnexinA5	GAPDH
slope	-3.321	-3.858
Y-Inter	40.06	38.27
R ²	0.99	0.97
Efficiency	1.99	1.97

The Comparative Ct method was used to compare AnnexinA5 gene expression between restenosis and non-restenosis group.

Δ Ct of each sample was calculated using the formula Ct (AnnexinA5) - Ct (GAPDH). The normality of Δ Ct distribution in case and control groups was measured using Kolmogorov-smirnov test, P value was <0.05 and distribution was out of the normal state, Mann-Whitney non-parametric test was used to compare the Δ Ct of the two groups and P value was 0.034 means that expression of anxa5 gene in ISR and NISR group was significantly different.

Fold change calculating:

To compare the difference of AnnexinA5 gene expression between case and control groups we used the formula $\Delta\Delta$ Ct = Δ Ct ISR – Δ Ct NISR.

Because of the approximately 100% efficiency of both primers (target and reference genes) in PCR, we used Livak method and $2^{-\Delta\Delta Ct}$ formula to calculate the fold change of AnnexinA5 gene expression in the ISR group in comparison to the NISR group. The fold change distribution was out of the normal state and the mean fold change was used to compare the change in gene expression, which was 0.05, meaning that the expression of the anxa5 gene in the ISR group was 50% lower than in the NISR group(Figure1).

Discussion

This study aimed to find the expression of AnnexinA5 in white blood cells (WBC) of in-stent restenosis (ISR) patients compared to WBC of the non-in-stent restenosis (NISR) control cases.

As it was expected based on AnnexinA5 anti-inflammatory action, the main result of this study has shown a significant reduction (p =0.034) in the expression of AnnexinA5 in WBC of the in-stent restenosis

patients compared to WBC of the non in-stent restenosis control cases. On the other hand, fold change calculations showed 50% reduction in the expression of AnnexinA5 in the ISR cases.

The role of AnnexinA5 as an anti-inflammatory agent has been shown in studies as there Ewing et al. illustrated that its administration could diminish macrophage and leukocyte cohesion which led to the decrease in atherosclerotic development, macrophage presence and GRP78(endoplasmic reticulum stress marker) (21, 25, 26). Our study was the first study on AnnexinA5 expression in WBCs and association to coronary restenosis. Ewing et al. examined the effect of systemic injection of AnnexinA5 on the development of neointima hyperplasia in hypercholesterolemic ApoE^{-/-} mice. After damaging the femoral arteries of the specimens and injection of systemic AnnexinA5, they sampled biopsy from artery and studied the tissue at the 3 days and 14 days later. Three days later, the presence of leukocytes in the tissue and the ratio of monocytes to macrophages in the group receiving AnnexinA5 was decreased, and AnnexinA5 was able to reduce the presence of leukocytes and inflammation. In the 14 days later, the thickness of the intima in the case group was about 60% less than in control group. Also, at this time, the expression of GRP78, was examined and its expression and, as a result, the inflammatory activity of cells were lower in the group receiving AnnexinA5 (21).

Vantits LJ, et al in a case – control study found that plasma levels of AnnexinA5 are inversely related to the severity of coronary stenosis and are indicative of the extent of restenosis, this fact indicates that the anxA5 could be considered as a marker for cardiovascular disease progression (18, 26).

De Jong RC et al, found that AnnexinA5 treatment reduces the inflammatory response ApoE*3-Leiden mice after MI-R injury (20).

Clausell et al. designed a study on atheroma tissue obtained from atherectomy of coronary artery 16 cases, patients with coronary restenosis had increased expression of the interleukin-1 and TNF α genes and also observed increasing in T lymphocytes in atheroma tissue (27).

Because of the anti-inflammatory role of AnnexinA5, we observed down regulated of AnnexinA5 expression in patients with coronary restenosis, our study is consistent with the results of other studies.

Considering the high cost and complexity of coronary artery restenosis treatment (28), if physicians being able to predict the risk of patients coronary restenosis based on their genetic backgrounds (precision medicine) they will be able to select the appropriate stent type or reasonable treatment (angioplasty or open heart surgery) for patient.

Declarations

Funding:

The authors would like to thank the Isfahan University of Medical Sciences, Isfahan, Iran for the financial support (Grant Number: 1141292).

Conflicts of interest/Competing interests:

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Availability of data and material: Not applicable

Code availability: Not applicable

Authors' contributions:

BM: contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript.

SSS: contributed in the conception of the work, conducting the study, data analysis for the work.

MS: contributed in the conception of the work, conducting the study, revising the draft.

MM: contributed in the conception of the work, conducting the study, data analysis for the work.

IO: contributed in the conception of the work, conducting the study, revising the draft.

RM: contributed in the conception of the work, conducting the study, revising the draft.

PA: contributed in the conception of the work, conducting the study, data analysis for the work.

GMM: contributed in the conception of the work, conducting the study, revising the draft.

SMH: contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

Ethics approval:

The study protocol (2015/930834) was approved by the Ethics Committee of the Medical University of Mashhad, Iran.

Consent to participate:

The Scientific Ethics Committee of the Medical University of Isfahan, Iran approved the study protocol (2015/930834) and all patients provided written informed consent.

Consent for publication:

All authors are satisfied with the publication of the article in the Molecular Genetics and Genomics journal

Acknowledgments:

We would like to thank Gholamrezapour, Asadi and technicians of AL-zahra genetic laboratory and catheterization lab nurses of Sina hospital, Iran. This study was supported by grants from medical university of Isfahan (No: 1141292) and medical university of Mashhad (No: 93570), Iran.

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Figures

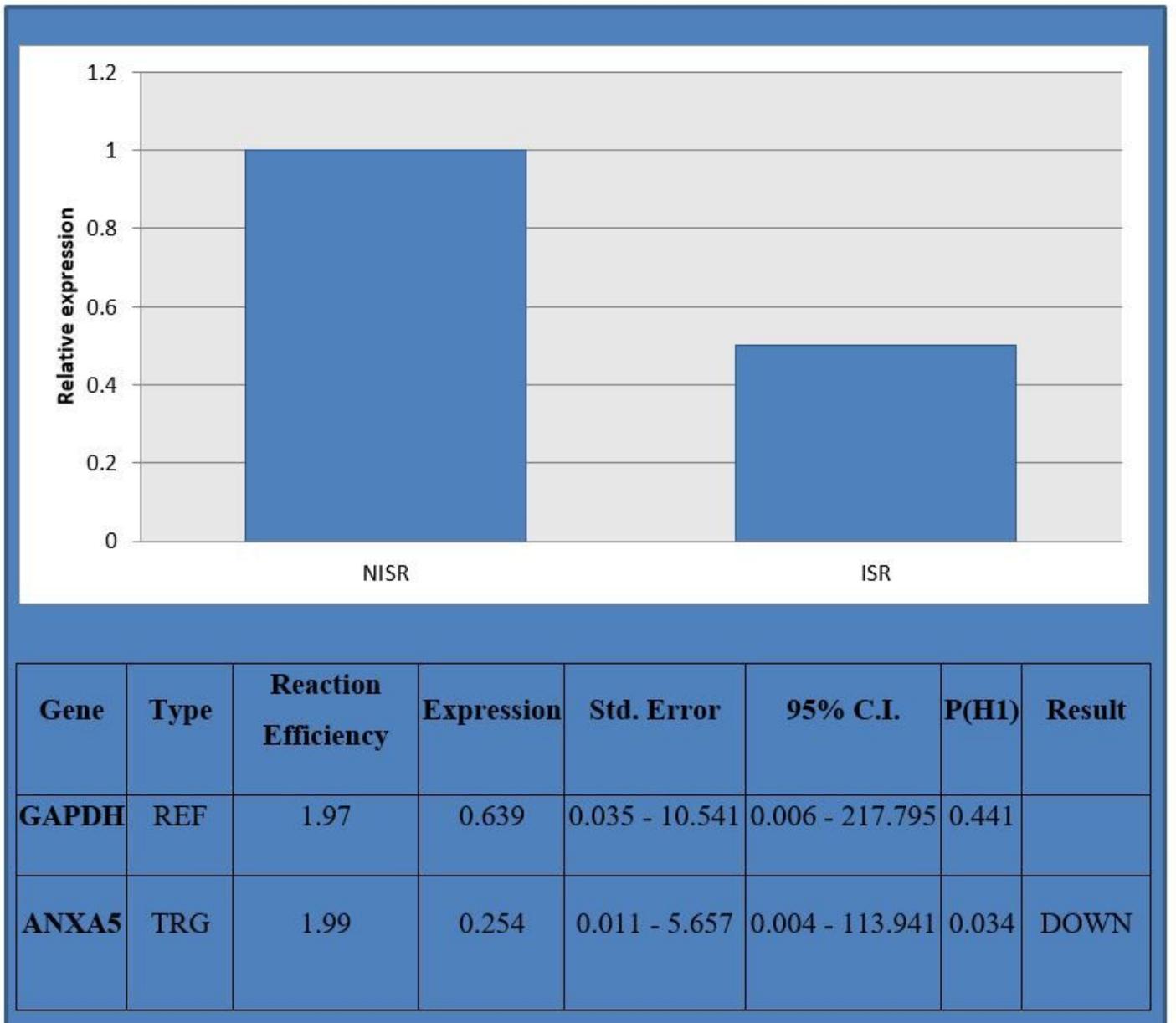


Figure 1

Relative quantification, ANXA5 gene expression in comparison to GAPDH reference gene expression