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ORIGINAL ARTICLE

TLR3 and TLR4 as potential clinical biomarkers for in-stent restenosis in drug-eluting stents patients

Shao Liang[1] · Ma Aiqun[3] · Li Jiwu[1] · Zhang Ping[2]

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Abstract In-stent restenosis is still a clinic trouble for percutaneous coronary intervention in drug-stent era. The molecular basis of restenosis is intensively associated with inflammation. TLR3 and TLR4 as innate immune factors have been proven to play a key role in atherosclerosis disease. The aim of this study is to study the TLR3 and TLR4 expressions and their downstream signaling proteins in the inflammatory process of restenosis after drug-stent therapy. mRNA and protein expression of TLR3 and TLR4 were detected in peripheral blood monocytes of primary group ($n = 38$), N-ISR group ($n = 36$) and ISR group ($n = 33$). Some inflammatory factors (including TLR3 and TLR4) were evaluated in serum of three groups. mRNA and protein expression of TLR3 and TLR4 and their downstream signaling proteins have shown a higher level in restenosis patients than non-restenosis patients and even primary patients who accepted first stent therapy. In serum, different from some nonspecific and downstream inflammatory factors, TLR3 and TLR4 also show a significantly higher level in ISR group compared with N-ISR group and primary group. This study provides a potential clinical biomarker for in-stent restenosis in drug-stent patients and some interesting data about the role of TLRs and their downstream signaling factors in the inflammatory process of in-stent restenosis. Compared with first stent therapy and non-restenosis patients, it is hopeful that TLR3 and TLR4 are potential noninvasive biomarkers in prognosis restenosis.

Keywords TLR · Inflammation · In-stent restenosis

✉ Shao Liang
shaoliang021224@hotmail.com

✉ Zhang Ping
zhangkiki520@126.com

[1] Department of Cardiology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China

[2] Department of Geriatrics and Neurology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China

[3] Department of Cardiovascular Medicine, First Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China

Introduction

Coronary artery atherosclerosis is a global burden disease. As a major treatment, percutaneous transluminal coronary intervention (PCI) preserves thousands of coronary artery disease patients [1]. In-stent restenosis (ISR) after stent deployment is still a clinical troublesome problem for percutaneous coronary intervention. Though drug-eluting stent gradually replaces bare-metal stent for angiographic and clinical good outcome, there is about 7–13 % risk of restenosis occurrence [2].

ISR mechanism comprises several processes which are smooth muscle cell modulation and proliferation, extracellular matrix remodeling and inflammation as the shown in researches of animal and human [3–5]. Inflammation and immune activation after stent-vessel injured take an important place in restenosis mechanism. Leukocyte accumulation, recruitment and infiltration cause a high odd occurrence at sites of stent invasion injury. After PCI operation, some inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), adhesion molecules and tumor necrosis factor (TNF) could induce endothelial regeneration and proliferation and result to restenosis after stent therapy [6, 7]. So drug-eluting stent are covered with one immunosuppress

drug coat (paclitaxel, everolimus or rapamycin) to inhibit smooth muscle cell modulation and proliferation [8–10].

As the most characterized innate immune receptors, Toll-like receptors (TLRs) which are expressed by leukocytes, dendritic cells (DCs) and T and B lymphocytes show a key role in coronary artery atherosclerosis disease and vascular injury [11, 12]. TLR4 could stimulate macrophages to directly induce atheroma formation, but TLR3 has a protective effect for vascular endothelium cell in arterial injury and atherosclerosis [13–15].

In our study, TLR3, TLR4 and their downstream signaling protein expression were evaluated in coronary artery atherosclerosis patients with or without in-stent restenosis compared with control population. The data aim for a novel view of innate immune response to in-stent restenosis and a potential noninvasive biomarker for in-stent restenosis.

Methods

Study population

All volunteers were recruited from the First Affiliated Hospital of Medical College of Xi'an Jiaotong University or the Second Affiliated Hospital of Wenzhou Medical University, followed the Declaration of Helsinki and approved by Institutional Ethics Committee of Xian Jiaotong University.

The subjects were divided into three groups: (a) primary group, who accepted first drug-stent (rapamycin or everolimus) therapy with only one vascular stenosis ($n = 38$); (b) N-ISR group ($n = 36$), who accepted drug-stent (rapamycin or everolimus) therapy in 8–12 months without angiographic restenosis; (c) ISR group, who accepted drug-stent (rapamycin or everolimus) therapy in 8–12 months with angiographic restenosis ($n = 33$). Inflammatory disease, autoimmune disease, acute fetal diseases, carotid or peripheral artery diseases and neoplastic diseases should be excluded for TLRs on that condition.

Before angiographic surgery, radial artery blood samples were collected.

TLRs mRNA analysis

In a two-steps method, mRNA expression of TLR3 and TLR4 was analyzed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), and β-actin was used for internal control.

SYBR® Premix EX Taq (TAKARA, Japan), 200 ng of cDNA template and a set of TLR primers were mixed as a reaction mixture for reverse primer.

The sequences of the forward primer and reverse primer in TLR3 were 5′-ATT AGG AAC TCA GGT TCA GC-3′ and 5′-GGA CAT TGTTCA GAA AGA GG-3′. The sequences of the forward primer and reverse primer in TLR4 were 5′-TGC GGG TTC TAC ATC AAA-3′ and 5′-CCA TCC GAA ATT ATAAGA AA AGT C-3′. The sequences of the forward primer and reverse primer of β-actin were 5′-AGC CTC GCC TTT GCCGA-3′ and 5′-CTG GTG CCT GGG GCG-3′.

Western blot analysis

Protein of each sample was abstract for peripheral blood monocytes. Protein was transferred to a polyvinylidene fluoride membrane and immersed in 5 % skim milk for 2 h. Anti-human TLR3 antibody (SC-16238, Santa, USA) diluted 1:1000, anti-human TLR4 antibody (bs3489, Bioworld, USA) diluted 1:1000, anti-human myeloid differentiation factor 88 antibody (MyD88, bs3521, Bioworld, USA) diluted 1:1000, anti-human TIR domain-containing protein-β antibody (TRIF-β, ab13810, Abcam, USA) diluted 1:1200, anti-human interferon regulatory factor 3 antibody (IRF3, ab21680, Abcam, USA) diluted 1:1200 and anti-human interferon-β antibody (INF-β, sc-73302, Santa Cruz, JAP) diluted 1:1200 was separately used to interacted with target proteins overnight at 4 °C room. Second antibody was used before immunoblotting exposure. β-Actin was used as loading and internal control.

Serum cytokines detection

Levels of cytokines TNF-α, IP-10, IL-8, IL-10, MCP-1, VEGF, TLR3 and TLR4 were also measured in the serum which was separated and frozen at 80 °C until assayed. This was performed using ELISA kits (TLR4 ELISA kit, ABIN414556, antibodies-online; TLR3 ELISA kit, ABIN417426, antibodies-online; Human MCP-1 TNF-alpha, IL-10, IL-8 and VEGF Quantikine ELISA kit, R&D Systems; IP-10 Human ELISA kit, KAC2361, Life Technologies).

Statistical analysis

All data were shown as mean ± SD for parametric value or as median and interquartile range for nonparametric value, and the comparisons of continuous data among groups were performed by one-way ANOVA followed by Scheffé's test in SPSS 13.0. $p < 0.05$ was considered as statistically significant difference.

Results

Baseline characteristics

The subject was divided into three groups as described in methods (Fig. 1). In Table 1, there was some baseline data

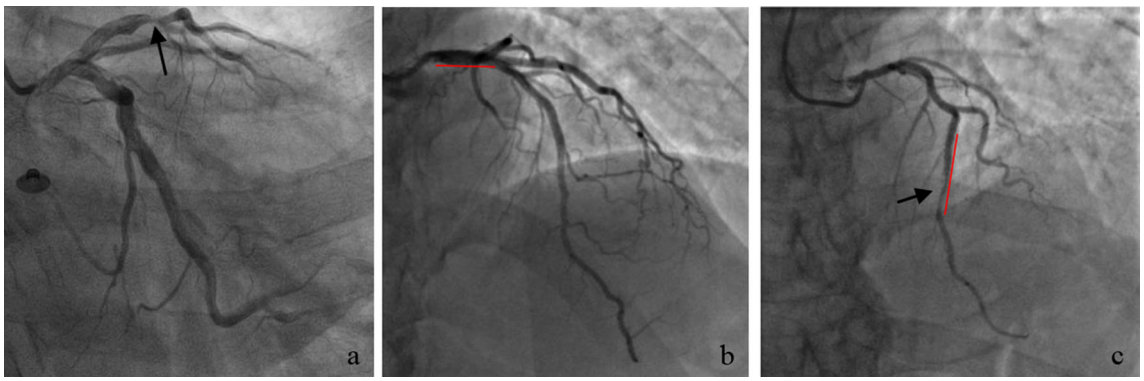


Fig. 1 Coronary angiography in each group. **a** primary group with one vascular stenosis; **b** N-ISR group without in-stent restenosis; **c** ISN group with in-stent restenosis. *Black line* (*red line* in online) shows the stent site, and *arrow* show the stenosis

Table 1 Subject characteristics

	Primary ($n = 38$)	N-ISR ($n = 36$)	ISR ($n = 33$)	p value
Age	59 ± 4	61 ± 5	58 ± 6	0.548
Gender (M/F)	17:18	17:19	15:18	0.631
Smoking	20 (52 %)	19 (53 %)	17 (51 %)	0.723
Drinking	22 (58 %)	21 (58 %)	18 (56 %)	0.717
Hypertension	30 (79 %)	28 (78 %)	26 (78 %)	0.875
Diabetes	12 (32 %)	13 (36 %)	11 (33 %)	0.792
TC (mmol/l)	6.13 ± 0.57	5.79 ± 0.44	6.78 ± 0.79	0.246
TG (mmol/l)	2.14 ± 0.58	1.98 ± 0.42	2.09 ± 0.37	0.526
HDL cholesterol (μmol/L)	1.03 ± 0.13	0.98 ± 0.17	1.14 ± 0.21	0.248
LDL cholesterol (μmol/L)	4.47 ± 0.73	4.13 ± 0.84	4.53 ± 0.91	0.425
Apolipoprotein A1 (g//L)	1.24 ± 0.16	1.15 ± 0.23	1.31 ± 0.33	0.627
Apolipoprotein B (g/L)	0.97 ± 0.27	1.03 ± 0.41	0.89 ± 0.21	0.579
cTnI (ng/ml)	1.97 (0.89, 2.34)	0.84 (0.49, 1.25)	2.04 (1.02, 2.21)	<0.05
CK-MB (IU/L)	14.23 (10.22,18.45)	12.46 (12.71,17.75)	13.78 (13.41, 18.26)	0.278
hs-CRP (mg/L)	7.43 (5.65, 9.76)	5.12 (3.32, 6.45)	8.43 (6.11, 10.63)	<0.05
LDH (IU/l)	122.44 ± 18.47	118.29 ± 20.35	141.91 ± 23.31	0.212
BUN (mmol/L)	6.12 ± 0.67	5.85 ± 0.74	6.07 ± 0.43	0.385
SCr (μmol/L)	71.16 ± 6.35	69.55 ± 8.42	70.21 ± 7.47	0.427
MDRD eGFR (ml/min/1.73 m^2)	66.77 ± 8.29	70.21 ± 9.17	62.53 ± 7.38	0.673
Medication				
Aspirin	38 (100 %)	36 (100 %)	33 (100 %)	1
Plavix	38 (100 %)	36 (100 %)	33 (100 %)	1
Statins	38 (100 %)	36 (100 %)	33 (100 %)	1
ACEI	21 (55 %)	18 (50 %)	20 (60 %)	0.134
β-blockers	22 (58 %)	21 (58 %)	20 (60 %)	0.558

Values are mean ± SD or n (%) or median and interquartile range

TC total cholesterol, *TG* triglycerides, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *cTnI* cardiac troponin I, *CK-MB* creatine kinase isoenzyme MB, *hs-CRP* high-sensitivity C-reactive protein, *BUN* blood urea nitrogen, *SCr* serum creatinine, *MDRD eGFR* modification of diet in renal disease-estimated glomerular filtration rate, *ACEI* angiotensin-converting enzyme inhibitor

about the subjects. There was no significant difference among three groups on age, gender, previous disease history, TG, TC and LDL, etc. And in medication, there was also no obvious difference in three groups. However, cTnI and hs-CRP had shown significant difference in three groups.

TLR3 and TLR4 mRNA expressions

As shown in Fig. 2, TLR3 of ISR group was significantly lower than that of primary group and N-ISR group ($p < 0.05$), and TLR3 of primary group was also obviously lower compared with N-ISR group ($p < 0.05$). In contrast, TLR4 of N-ISR group was the highest one in three groups ($p < 0.05$), and TLR4 of ISR was significantly higher than that of primary group ($p < 0.05$).

TLRs and downstream signaling proteins expression

In Fig. 3a, TLR3 and TLR4 protein expressions were estimated in peripheral blood monocytes. There was a similar tendency according to their mRNA expression. TLR3 protein expression was highest in N-ISR group ($p < 0.05$), but lowest in ISR group ($p < 0.05$). In contrast, TLR4 protein expression was highest in ISR group ($p < 0.05$), but lowest in N-ISR group ($p < 0.05$).

In Fig. 3b, MyD88 and TRIF-β as downstream signaling proteins of TLR4 were highest in ISR group ($p < 0.05$), but lowest in N-ISR group ($p < 0.05$).

As downstream signaling protein of TLR3, the expression of IRF3 and IFN-β was similar in three groups. In ISR group, IRF3 and IFN-β protein expressions were significantly higher than that of N-ISR group and primary group ($p < 0.05$). But IRF3 and IFN-β protein expressions of N-ISR group were obviously lower than that of primary group ($p < 0.05$; Fig. 3c).

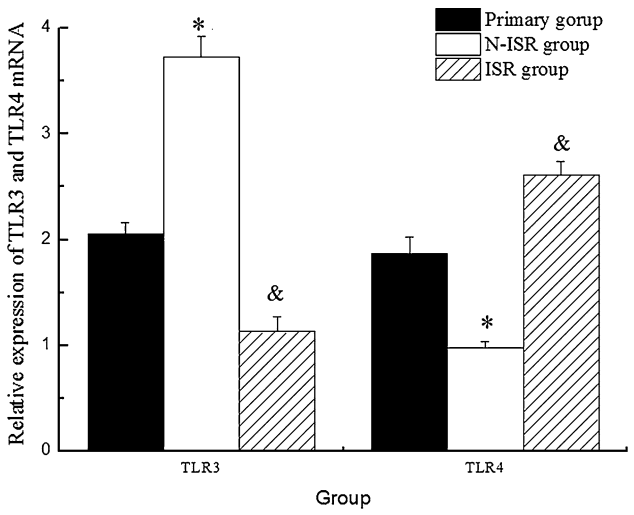


Fig. 2 TLR3 and TLR4 mRNA expression levels in mononuclear cells. *TLR* Toll-like receptor, *ISR* in-stent restenosis. $^{*}p < 0.05$ compared with primary group and ISR group; $^{\&}p < 0.05$ compared with primary group and N-ISR group. $n = 38$ in primary group, $n = 36$ in N-ISR group, $n = 33$ in ISR group

Fig. 3 TLRs and downstream signal proteins expression. TLR Toll-like receptor, MyD88 myeloid differentiation factor 88, TRIF-β TIR domain-containing protein-β, IRF3 interferon regulatory, IFN-β interferon β. $^{*}p < 0.05$ compared with primary group and ISR group; $^{\&}p < 0.05$ compared with primary group and N-ISR group. $n = 38$ in primary group, $n = 36$ in N-ISR group, $n = 33$ in ISR group

Serum inflammatory factor expression

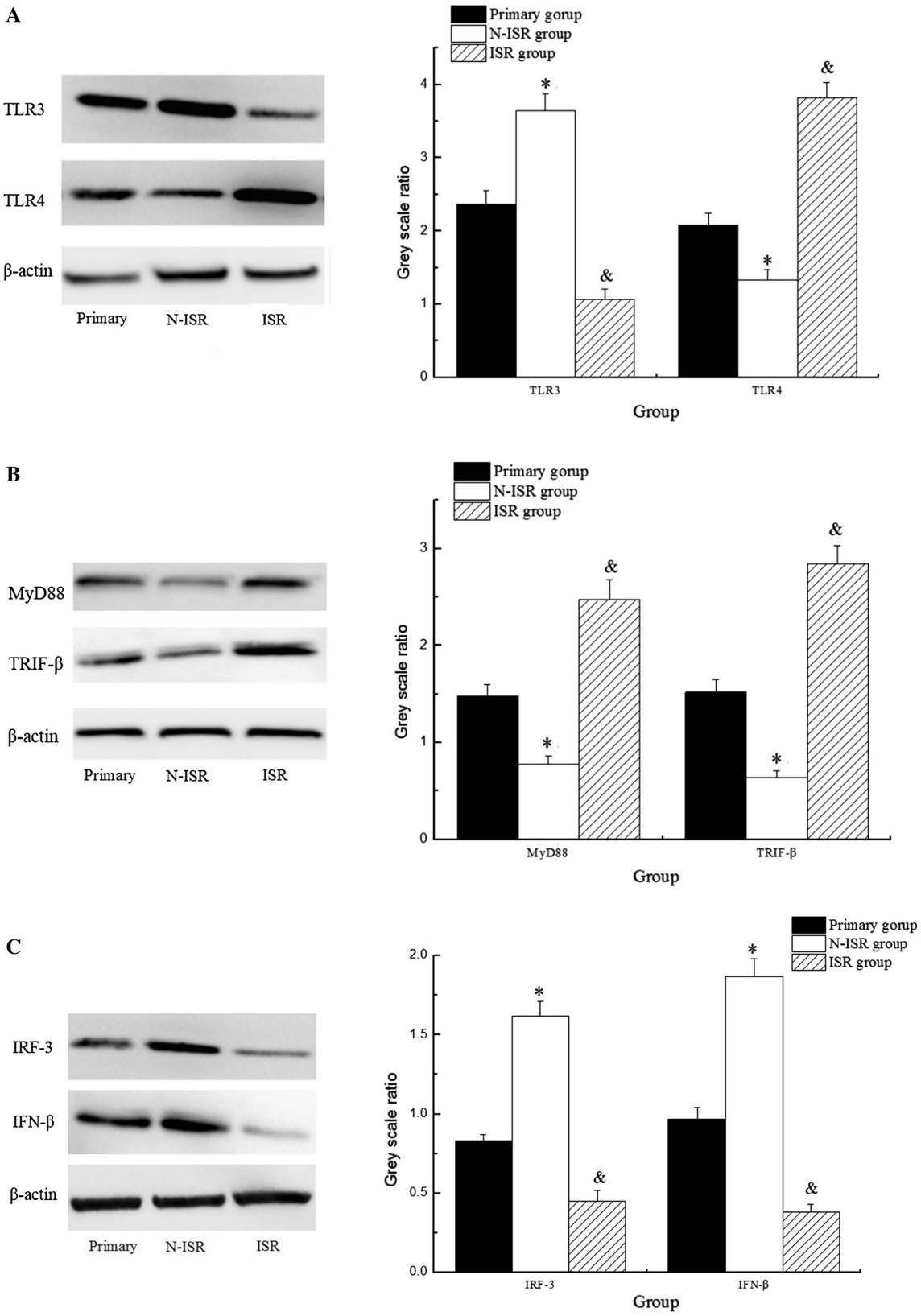
There were several serum inflammatory factors detected. As shown in Table 2, MCP-1, VEGF, IL-8 and TLR4 of N-ISR group were significantly lower than that of primary group and ISR group ($p < 0.05$), but IP-10, IL-10 and TLR3 were significantly higher than that of primary group and ISR group ($p < 0.05$). Notably, serum TLR3 expression of ISR was obviously lower than that of primary group ($p < 0.05$), but serum TLR4 expression of ISR was obviously higher than that of primary group ($p < 0.05$). However, there was no significant difference between ISR group and primary group in MCP-1, VEGF, IL-8, IP-10 and IL-10 ($p < 0.05$).

Discussion

In this study, we examined mRNA and protein expression of TLR3 and TLR4, downstream signaling protein and some serum inflammatory factors in drug-stent patient with or without restenosis. TLR3 and TLR4 were appointed for novel clinical biomarker as restenosis risk in drug-stent patients.

The potential mechanism is complex and obscure, but most of researches focus on the inflammatory response. In animal study, macrophages accumulation and leukocyte migration were observed around stent struts in days or weeks later after stent treatment [16, 17]. MCP-1, VEGF and some other inflammatory factors were detected to increase after injury, so targeting anti-MCP-1 (or other inflammatory factors) shows a benefit effect to reduce aortic lipid content and monocyte accumulation, diminish neointimal thickening, and attenuate atherogenesis [18–20]. In recent research, TLRs have been proven to regulate MCP-1 and VEGF expression via downstream signaling pathway [21, 22].

TLRs as innate immune receptors take a key role in inflammation mediation and inflammation-associated diseases. TLR4 has a harmful effect as stimulation of macrophages via MyD88- and TRIF-β-dependent pathways to atheroma formation [23, 24]. In contrast, TLR3 shows a protective role in vascular injury via IFN-β and IRF-3 [25, 26]. In our study, mRNA and protein expression of TLR4 and downstream signaling proteins have shown a higher level in restenosis patients than non-restenosis patients and even primary patients who accepted first stent therapy. In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IRF-3
IFN-β
Group

Table 2 Serum inflammatory factor expression

	Primary ($n = 38$)	N-ISR ($n = 36$)	ISR ($n = 33$)
TLR3 (pg/ml)	179.17 ± 15.09	278.43 ± 17.53*	112.37 ± 10.22[&]
TLR4 (pg/ml)	492.71 ± 17.36	313.9 ± 15.68*	645.17 ± 21.75[&]
MCP-1 (pg/ml)	791.37 ± 56.52	414.15 ± 49.26*	811.07 ± 65.13
VEGF (pg/ml)	326.74 ± 20.14	174.31 ± 18.11*	354.29 ± 19.07
TNF-α (pg/ml)	42.84 ± 5.17	21.22 ± 3.51*	39.19 ± 4.63
IP-10 (pg/ml)	172.54 ± 20.07	317.31 ± 19.24*	227.19 ± 21.25
IL-8 (pg/ml)	32. 33 ± 6.51	52.31 ± 6.15*	38.37 ± 5.96
IL-10 (pg/ml)	11.31 ± 1.72	37.36 ± 1.63*	12.24 ± 2.08

TLR Toll-like receptor, *MCP-1* monocyte chemotactic protein 1, *VEGF* vascular endothelial growth factor, *TNF-a* tumor necrosis factor-a, *IP-10* interferon-induced protein 10, *IL-8* interleukin-8, *IL-10* interleukin-10

*$p < 0.05$ compared with primary group and ISR group; [&] $p < 0.05$ compared with primary group and N-ISR group

serum, different from some nonspecific and downstream inflammatory factors, TLR4 also shows a significantly higher level in ISR group compared with N-ISR group and primary group. But for TLR3, there is a lower level in ISR group compared with N-ISR group and primary group from mRNA to protein and serum cytokine.

As shown in past studies, there is no report about β-blockers, aspirin or plavix on TLRs and their pathway proteins expression,but ACE inhibitor and statins could decrease the TLR4 expression [27–29]. However, there is no different ACE inhibitor and statins therapy in three groups.

In summary, this study provides a potential clinical biomarker for in-stent restenosis in drug-stent patients and some interesting data about the role of TLRs and their downstream signaling factors in the inflammatory process of in-stent restenosis. Compared with first stent therapy and non-restenosis patients, it is hopeful that TLR3 and TLR4 are potential noninvasive biomarkers in prognosis restenosis.

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Compliance with Ethical Standards

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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