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Effect of YKL-40 RNA Interference on VEGF Gene Polymorphism Expression in Atherosclerotic Mice

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Abstract: **Aims:** To investigate the effect of YKL-40 RNA interference on VEGF gene polymorphism expression in atherosclerotic mice. **Methods:** After the atherosclerosis models in mice were built, the mice were divided into three groups including control group, negative control group and observation group, which were separately given to normal saline, negative virus (5×10^7 TU) and YKL-40 RNA interference lentivirus. Then the whole blood DNA was extracted and genotyped in each group of mice and the expression of VEGF in each group of mice was detected by PCR, while the expression level of inflammatory factors in each group of mice was detected by ELISA. Meanwhile, the aortas of mice in each group were pathologically analyzed and the atherosclerosis of mice was detected. **Results:** Compared with the control group, the VEGF content in both the virus negative control group and the observation group was significantly increased（$P<0.05$）. The detection rates of CC genotype and C allele at rs699947 of VEGF gene in the observation group were significantly higher than those in the control group and the virus negative control group, and the difference was statistically significant. There were no significant changes for the expression of HDL-C, LDL-C, TC and TG in mice of each group（$P>0.05$）. Moreover, the levels of Lp-PLA_2 and MCP-1 in the negative control group were significantly increased ($P < 0.05$), while those in the observation group were significantly decreased ($P < 0.05$) compared to that in control group. What's more, the histomorphology of the observation group was significantly different from that of the control group and the virus negative control group. The thickness of the fibrous cap of the as plaque was significantly higher than that of the control group and the virus negative control group, but the plaque area and fat content were significantly lower than that of the control group and the virus negative control group and the NC group. Besides, there was no significant difference in lipid content, fiber cap thickness and plaque area between the control group and the virus negative control group. **Conclusion:** YKL-40 RNAi could improve the VEGF polymorphism, reduce the expression of LP- PLA_2 and MCP- 1, and significantly inhibit the occurrence and development of atherosclerosis, which was expected to provide a new target for the prevention and treatment of atherosclerosis.

Keywords: YKL-40; VEGF; Single Nucleotide Polymorphism

Introduction

Atherosclerosis (AS) is a chronic progressive vascular disease, which is an important cause of global cardiovascular disease and the main cause of death [1] and is often found in large and medium arteries and is characterized by lipid accumulation and inflammation [2]. A variety of inflammatory cells participate in the occurrence and development of AS [3]. In recent years, the discovery of a large number of as inflammatory factor markers has provided new ideas for the early diagnosis of as, and also provided new targets and new directions for the treatment of diseases [4]. However, the interaction between various inflammatory factors needs further study in the process of atherosclerosis.

Vascular endothelial growth factor (VEGF) is a heparin binding protein family that participates in angiogenesis, lymphopoiesis and lymphangiogenesis, resists oxidative stress, regulates lipid metabolism and inflammation [5, 6]. Studies have found that there are more than 30 single nucleotide polymorphisms (SNPs) in VEGF. These gene polymorphisms affect

the expression and transcription of VEGF, and then affect its role. The polymorphism analysis of VEGFR gene showed that two single nucleotides in the promoter region and coding region of VEGFR-2 gene: rs2071559 (- 604T / C) and rs1870377 (+ 1719a / T) were associated with the risk of peripheral arterial disease. VEGF is a powerful mitogen, which can promote the proliferation and migration of endothelial cells and the formation of new blood vessels[7], and plays an important role in the development of as and the stability of plaque. The gene polymorphism of VEGF and its receptor leads to the difference of expression and affects the physiological efficacy of VEGF. Other studies have shown that YKL- 40 is highly expressed in AS [8]. The aim of this study was to study the changes of VEGF gene polymorphisms in the process of atherosclerosis in apoE -/-mice interfered by YKL-40, and to elucidate the interaction between YKL-40 and VEGF in AS.

1. Matrials and methods

1.1 Experimental animals and reagents

A total of 72 male apoe-/- mice of 12-week-old were purchased from the medical department of Peking University, which were housed in IVC-II isolation cages and fed with high fat at the atmosphere of 20-25 °C, 55 ± 5% of relative humidity, 20-50 PA pressure and 12 hours light / dark cycle. The experiment was approved by the Ethics committee of Jinan People's Hospital Affiliated to Shandong First Medical University, and conform to the guidelines for the care and use of experimental animals of the National Institutes of health and the ARRIVE guidelines.

The construction of YKL-40 shRNA lentiviral vector (target gene sequence: 5 ′ - gctccagtgctgcata-3 ′) was completed by Shandong Weizhen Biotechnology Co., Ltd. Trizol reagent was purchased from GIBCO (USA), while ELISA kits such as oil red O, monocyte chemotactic protein-1 (MCP-1) and lipoprotein associated phospholipase A 2 (Lp-PLA 2) were purchased from Zhengzhou sensike Biological Products Co., Ltd. Besides, ELISA kits such as mouse vascular endothelial growth factor (VEGF), mouse high density lipoprotein cholesterol (HDL-C), mouse low density lipoprotein cholesterol (LDL-C), mouse triglyceride (TG) and mouse total cholesterol (TC) were purchased from Shanghai Kexing Trading Co., Ltd.

1.2 Establishment of mouse AS model and animal grouping

The AS model of apoe-/- mice was established by inducing the formation of as with the left common carotid artery constriction cannula method and kept high-fat feeding. 8 weeks later, the apoE-/-mice were randomly divided into control group（n=24）, negative control group（NC, n=24）and observation group（n=24）. In the control group, the left common carotid artery was isolated and the silicone cannula was removed, and then saline was infused locally; The negative control group was injected with negative virus (5×10^7 TU）； The observation group was given YKL-40 RNA interference lentivirus (5×10^7 TU). Plaques were collected after 6 weeks to make 6 μ M U.M frozen sections, which were then stained with HE and oil red O and analyzed by histopathology.

1.3 Whole blood DNA extraction and genotyping

200 ml of blood cells were collected and the genomic DNA was extracted according to the instructions of UNIQ-10 column clinical sample genome extraction kit, and the colorimetric quantification and purity identification were conducted with ultraviolet spectrophotometer. The extracted DNA was stored in a refrigerator at 4 ° C or -20 ° C. Genotyping was performed by polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP) and sequence-based typing (SBT). The DNA fragment containing VEGF rs699947-471f was PCR amplified. The primer sequence was synthesized by Shanghai Sangong Bioengineering Co., Ltd., and the amplified PCR product was recovered and subjected to first-generation sequencing (Sanger sequencing).

1.4 Fluorescence quantitative PCR

10 mg of sample, 200 μ L buffer SA and 10 μ L proteinase K was put into the centrifuge tube and mixed well, which was then incubated at 56 ° C for 10 minutes, treated at 95 ° C for 5 minutes, and centrifuged for 5 minutes at 13000 RPM (~

17900 × g). The supernatant was taken into a new centrifuge tube for PCR amplification with the reaction procedure in Table 1. Finally, quantitative PCR analysis was performed.

Table 1. The reaction conditions of PCR

Procedure	Temperature	Time	
Pre-denaturation	94℃	2min	
Denaturation	94℃	30s	30-40 cycles
Annealing	55-65℃	30s	
Extension	72℃	60s	
Final extension	72℃	5min	

1.5 ELISA analysis

The blood was taken from the anesthetized mice, and the upper plasma was taken after centrifugation for detection. The levels of VEGF, Lp-PLA $_2$, MCP-1, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG) and total cholesterol (TC) were determined using the corresponding ELISA kits.

1.6 Histomorphological examination

Image Pro Plus 5.0 image analysis software was used for quantitative analysis of HE stained and oil red O-stained sections, and the plaque area, fiber cap thickness and lipid content were detected.

1.7 Statistical analysis

The data were analyzed using SPSS 21.0 software. All measurement data were expressed as mean ± SD. After the normality test, one-way analysis of variance (ANOVA) was performed. Student Newman Keuls (SNK) test was used for comparison between two groups. If $P < 0.05$, the difference was considered statistically significant.

2. Results

2.1 Comparison of VEGF content and site polymorphism in mice of each group

Compared with the control group, the VEGF expression was significantly increased in both the NC group and the observation group ($P<0.05$, Fig. 1). According to the sequencing results, we found that the detection rates of CC genotype and C allele at rs699947 of VEGF gene in the observation group were significantly higher than those in the control group and the NC group ($P < 0.05$, table 1).

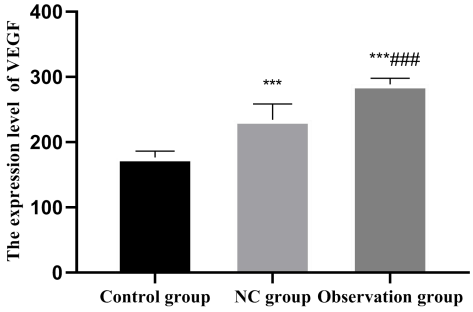

Figure 1. The expression of VEGF in each group，*P<0.05，**P<0.01，***P<0.001, vs control group；#P<0.05，##P<0.01，

###P<0.001, vs NC group.

Table 2. Comparison of polymorphisms of VEGF gene rs699947

	Genotype frequency			Allele frequency	
	CC	CT	TT	C	T
Control group（n=24）	13（54.2%）	7（29.2%）	3（12.5%）	17（70.8%）	6（25.0%）
NC group（n=24）	14（58.3%）	6（25.0%）	4（16.7%）	18（79.2%）	5（20.8%）
Observation group（n=24）	16（66.7%）	8（33.3%）	1（4.17%）	20（83.3%）	4（16.7%）

2.2 The expression of HDL-C, LDL-C, Lp-PLA2, MCP-1, TC and TG levels in each group

As shown in Figure 2, there was no significant change in HDL-C, LDL-C, TC and TG in mice of each group (P>0.05). Compared with the control group, the levels of Lp-PLA$_2$ and MCP-1 in the virus negative control group were significantly increased (P<0.05), while the levels of Lp-PLA$_2$ and MCP-1 in the observation group were significantly decreased (P<0.05).

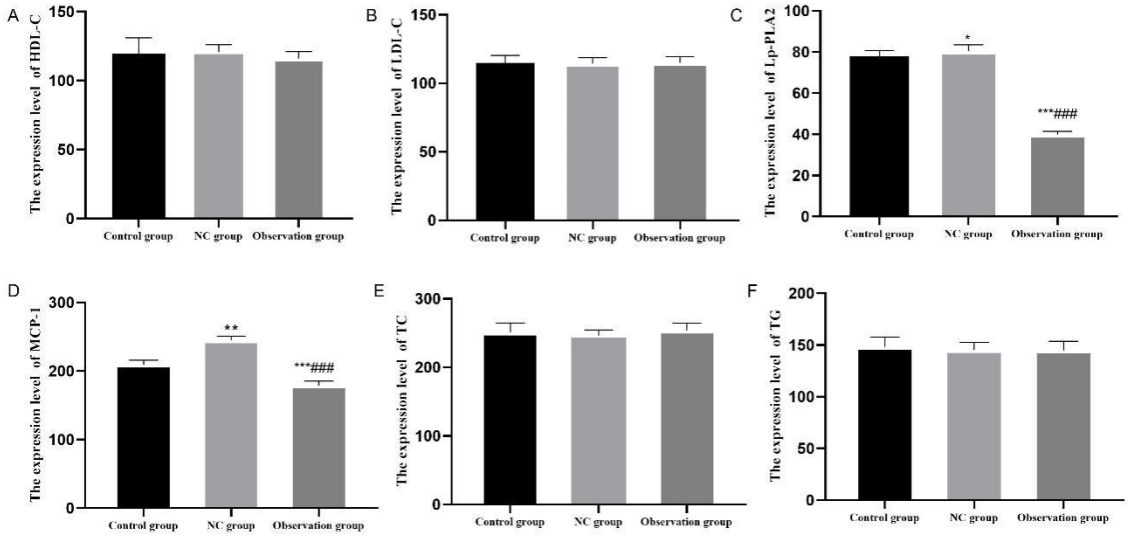

Figure 2. The expression of HDL-C, LDL-C, Lp-PLA2, MCP-1, TC and TG levels in each group. * P<0.05，**P<0.01，***P<0.001, vs Control group；#P<0.05，##P<0.01，###P<0.001, vs NC group.

2.3 Morphological changes of mice in each group

The histomorphology of the nice in observation group was significantly different from that of the control group and the NC group. The thickness of the fibrous cap of as plaque was significantly higher than that of the control group and the virus negative control group (P<0.05, Fig. 2), but the plaque area and fat content were significantly lower than that of the control group and the virus negative control group (NC group) (P<0.05, Fig.3). There was no significant difference in lipid content, fiber cap thickness and plaque area between the control group and the NC group, which indicated that the beneficial effect of the observation group was not caused by the non-specific immune response caused by virus infection, which further confirmed that the interference of RNAi was effective.

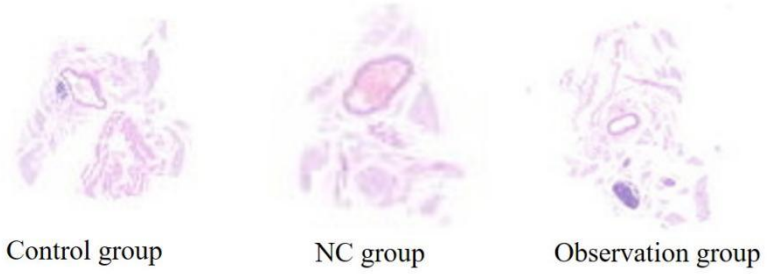

Figure 3. Morphological changes of mice in each group

3. Discussion

AS is a lipid driven chronic inflammatory disease that usually forms plaques in large and medium-sized arteries [9], and is the main cause of ischemic heart disease and stroke[10]. AS has a serious impact on people's quality of life and health, and has become a hot topic in the cardiovascular field.

As a growth factor, VEGF is widely expressed in normal and pathological tissues and plays an important role in angiogenesis [11]. Studies have shown that VEGF-A is related to the proliferation of vascular endothelial cells and the prevention of atherosclerotic plaque formation [12], and VEGF can induce the neovascularization of atherosclerotic plaques and the expansion of aortic calcification lesions [13]. In addition, VEGF protein may play an important regulatory role in the pathogenesis of endothelial dysfunction and atherosclerosis by mediating intimal hyperplasia, thereby improving the progression of atherosclerotic plaques in coronary arteries in human and animal models. VEGF expression is regulated by some single nucleotide polymorphisms (SNPs), some of which, including VEGF – 2578a/c (rs699947), - 1154g/a (rs1570360), + 405c/g (rs2010963) and + 936c/t (rs3025039), are associated with coronary artery disease susceptibility. However, these findings are controversial. This study found that VEGF – 2578a / C (rs699947) was related to the occurrence and development of atherosclerosis, and the expression of VEGF was decreased in AS. This was consistent with previous research results, which might be a manifestation of rs699947's protective effect on coronary artery disease [14].

YKL-40, also known as human cartilage glycoprotein-39 (HC-GP39), belongs to the mammalian 18 glycosylhydrolase family. As the immune inflammatory response theory of atherosclerosis is more and more accepted, the extensive role of YKL-40 in the inflammatory response suggests that it also plays an important role in the development of AS. It has been found that smooth muscle cells in carotid atherosclerotic plaques of some patients express YKL-40, and the high expression of YKL-40 mRNA can also be detected in giant cells in atherosclerotic plaques. However, the mechanism of YKL-40 in atherosclerosis is still unclear. In the early stage of AS, monocytes adhering to the vascular wall, under the action of monocyte chemoattractant protein-1 (MCP-1) produced by vascular endothelial cells and smooth muscle cells, migrate to the vascular wall along the concentration gradient, invade or activate into macrophages, and then combine with the modified LDL-C deposited in the vascular intima to form foam cells, thus forming atherosclerotic plaques. Studies have shown that YKL-40 can upregulate MCP-l expression, chemotactic more monocyte infiltration, and promote plaque formation and development. In vitro proteomic studies on biochemical indicators of AS indicated that the expression of YKL-40 in the supernatant of macrophages treated with oxidized low-density lipoprotein (ox LDL) was increased, which was similar to the formation of "foam cells", indicating that YKL-40 can promote the differentiation of monocytes into lipid loaded macrophages during the formation of atherosclerotic plaques. This was consistent with the findings that the atherosclerotic vascular smooth muscle cells also express YKL-40 protein in vivo. In this study, YKL-40 was interfered and its expression level of inflammatory factors was detected. The results showed that YKL-40 RNAi had no significant effect on the expression levels of HDL-C, LDL-C, TC and TG in mice, but could significantly reduce the expression of Lp-PLA_2 and MCP-1, indicating that YKL- 40 RNA interference reduces plaque lipid content and reduces plaque vulnerability by reducing the levels of inflammatory factors such as LP- PLA_2 and MCP- 1. In addition, the pathological experiments also showed that YKL-40 RNAi had obvious inhibitory effects on plaque formation, plaque fiber cap thickness, plaque area and

plaque fat content, which indicated that ykl-40rnai was effective in interfering with atherosclerosis.

In conclusion, YKL-40 RNAi could improve VEGF polymorphism, reduce the expression of LP- PLA_2 and MCP- 1, and significantly inhibit the occurrence and development of atherosclerosis. It is expected to provide a new target for the prevention and treatment of AS. However, this study also had some limitations, the number of animals used was relatively small, and further validation and mechanism research are needed in the later stage.

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