Hypomethylation of The Low-density Lipoprotein Receptor Class A Domain Containing 4 Gene in Rheumatoid Arthritis in a Chinese population

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Abstract

Background: Rheumatoid arthritis, a chronic inflammatory disease, is characterized by destruction of synovial joints. The pathogenesis of RA is unclear, and DNA methylation plays important roles in the expression of related genes. LDLRAD4 is identified from three GWAS, in which the expression of LDLRAD4 was different in RA. LDLRAD4functions as a negative regulator of TGF-β signaling, which affects cell proliferation, differentiation and apoptosis. There are few studies on the correlation between LDLRAD4 methylation and RA, and this study aims to investigate the relationship in Chinese population.

Methods and Results: Clinical information and peripheral blood samples of 150 RA and 150 healthy controls were collected, and DNA methylation levels of LDLRAD4 gene and CpG sites were detected. Hypomethylation of LDLRAD4 genewas observed in RA patients, especially LDLRAD4-43F and LDLRAD4-44F regions. The AUC value of LDLRAD4-44F region was 0.78 to predict disease. In RF+/CCP- group, methylation levels of LDLRAD4, LDLRAD4-42-1F, LDLRAD4-43F and LDLRAD4-44F regions were strongly correlated withDAS28, ESR and CRP, while in RF-/CCP+ group, LDLRAD4-42-2F region was strongly correlated with ESR and CRP.

Conclusions: LDLRAD4 methylation is a potential diagnostic and inflammatory indicator of RA.

1. Introduction

Rheumatoid arthritis(RA) is an autoimmune disease, characterized by erosive arthritis, which can occur at any age. The basic pathological manifestations are synovitis, pannus formation, and the destruction of articular cartilage and bone, leading to joint deformity and functional disability. RA can complicate with lung diseases, cardiovascular diseases, malignant tumors, depression, etc. In industrialized countries, RA affects 0.5-1%[1] of adults. The pathogenesis of RA is still unclear, and the agreement between identical twins is only 12−15%[2, 3], suggesting that epigenetics and environmental factors play important roles in the pathogenesis of RA. This study observed that the level of Low-density lipoprotein receptor class A domain containing 4 (LDLRAD4)methylation was lower in a Chinese RA population.

Epigenetics may be involved in the pathogenesis of RA by increasing the expression of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6. Epigenetic therapies that control systemic inflammation may be an important therapeutic target for RA, for epigenetics is reversible compared to genetic mutations. Three epigenetic mechanisms have been described: non-coding RNA, histone modification, and DNA methylation. DNA methylation is the most widely studied epigenetic mechanism, for it can be inherited through multiple cell division and is more stable than RNA and proteins(2). Kazuhisa Nakano[3] isolated 1859 differential DNA methylation loci in 6 RA and 5 osteoarthritis FLS lines, and hypomethylation loci were identified in the key RA-related genes, such as CHI3L1, CASP1, STAT3, MAP3K5, MEFV and WISP3, while hypermethylation loci include TGFBR2 and FOX01 were identified, suggesting that these genes may alter FLS gene expression and participate in the pathogenesis of RA. DNA methylation inhibitors, including azacytidine, decitabine, procainamide, and hydrazine, have been reported in RA[2]. DNA

methylation may be a biomarker of RA activity and also provide new evidence for the use of DMARDs and biologics in the treatment of RA [2, 4, 5].

The research sifted through three Genome-wide Association Studies(GCST002318, GCST90013534, GCST90018690), and LDLRAD4 was identified for its different expression in RA. LDLRAD4, also known as C18orf1, is located on chromosome 18, which has been previously proved to be associated with mental disorders(6). LDLRAD4 is also expressed in multiple organs, including immune organs (lymph nodes, spleen, etc.), and functions as a negative regulator of transforming growth factor β (TGF-β) signal, which affects cell proliferation, differentiation and apoptosis in RA. LDLRAD4 has been confirmed to be related with psychiatric diseases, malignant tumors, cerebral hemorrhage, chronic periodontitis, but there are few studies focused on the relationship between LDLRAD4 gene and RA. The lower level of LDLRAD4 methylation was observed in a Chinese RA population, providing an evident for disease diagnosis, disease activity, and potential therapeutic targets.

2. Materials and Methods

2.1 Participants

A total of 150 RA patients and 150 healthy controls matched with age and gender, were prospectively recruited from the Department of Rheumatology at the Qilu Hospital of Shandong University in Qingdao, China from 2017.1 to 2022.12. The clinical information (including age, gender, height, weight, body mass index (BMI), primary diseases, cigarette, alcohol, blood routine examination, hepatic and renal function, glucose, erythrocyte sedimentation rate(ESR), c-reactive protein（CRP）, DAS28 score, rheumatoid factor(RF), anti-cyclic citrullinated peptide antibody (CCP), lung manifestations, bone mineral density) were recorded and whole blood samples were collected. All 150 RA patients met the 2010 Rheumatoid Arthritis Classification Criteria of the American College of Rheumatology (ACR)（7）. This study protocol was reviewed and approved by Ethics Committee of Qilu Hospital of Shandong University（Qingdao） and was conducted in accordance with the 1964 Helsinki Declaration.

2.2 DNA Extraction and Quality Control

Whole blood samples were drawn using EDTA tubes, and methylation of the LDLRAD4 in whole blood was extracted using MethylTarget (Genesky Biotechnologies Inc. Shanghai, China). According to the following steps:(1) Quality control: DNA integrity was tested using agarose gel electrophoresis (AGE). The quality of the DNA was then detected by a NanoDrop 2000 (NanoDrop Technologies, Wilmington, Delaware, USA). (2) Designation and optimization of primers. (3) Optimization of the multiplex PCR primer plate: Mix the optimized primers in step (2) into the multiplex PCR primer plate and adjust the composition and concentration of the primers in the multiplex PCR plate using capillary electrophoresis (CE). (4) Sulfite processed: DNA samples were modified using the sulfite EZDNA Methylation e kit (Zymo Research, USA). (5) Multiple PCR procedures for target fragments in the samples. (6) Adding special tag sequences: Using Index sequence primers, a special tag sequence compatible with the Illumina platform (Illumina, California, USA) was added to the end of the DNA library by PCR. (7) High-throughput

sequencing: The Index PCR products of all samples were mixed in the same amount, and the final sequence library was obtained after tapping. Verify fragments length using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). High-throughput sequencing was performed at Illumina Hiseq (Illumina, California, USA) using a 2×150bp paired end sequencing mode after ensuring the molar concentration of the library. Sequencing depth 1000X.

2.3 CpG Island Selection

CpG islands located in the proximal of the LDLRAD4 gene were selected according to the following criteria: (1) 200-bp minimum length; (2) 50% or higher GC content; and (3) 0.60 or higher ratio of observed/expected dinucleotide CpG. Four regions from CpG islands of the LDLRAD4 gene were selected and sequenced.

2.4 Data analysis

Statistical analysis was performed using IBM SPSS27.0 software (IBM, Armonk, New York, USA). Continuous variables were expressed as mean ± standard deviation, and categorical variables were expressed as number and percentage. For normally distributed continuous variables with homogeneity of variance, the T-test was used to compare the mean value difference between two groups, otherwise the Wilcoxon test was used. ANOVA was used to compare the mean value among multiple groups. Chi-square test was used to compare the frequency distributions for categorical variables. Stepwise regression analysis was used to screen the variables, build a predictive model and plot the ROC curve. P value < 0.05 was considered statistically significant.

3. Results

The whole-blood LDLRAD4 methylation was detected in 300 subjects in this study, including 150 RA patients and 150 healthy controls. The average age of RA group was 57.28 ± 12.27 years old, with a male to female ratio of 1:3.55, and the average age of the control group was 56.16 ± 13.42 years old, with male to female ratio of 1:3.69. The basic information of the RA and control group is shown in the following table (Table 1). And 89 CpG sites in four regions from CpG islands of the LDLRAD4 gene were selected and sequenced(Fig. 1, Schedule 1).

Table 1
The clinical characters of RA and control group

clinical characteristics	RA	Control
Age(y)	57.28 ± 12.27 (150)	56.16 ± 13.42 (150)
Female	117 (150)	118 (150)
Course(y)	7.45 ± 8.74(150)	
BMI(kg/㎡)	23.28 ± 3.60(70)	23.63 ± 3.22(134㎡)
ESR(mm/h)	40.64 ± 25.33(150)	
CRP(mg/L)	29.89 ± 35.02(149)	
HB(g/L)	114.56 ± 18.85(150)	137.53 ± 16.88(140)
WBC(*10^9/L)	7.04 ± 2.43(150)	6.04 ± 1.40(140)
PLT(*10^9/L)	295.57 ± 105.74(150)	240.63 ± 49.48(140)
RF	109(150)	
CCP	112(150)	
DAS28	4.90 ± 1.16(150)	
IPF	18(150)	
Osteoporosis	52(150)	

The data were showed as mean ± sd or number, and (number) showed total number with available data.BMI body mass index, ESR erythrocyte sedimentation rate, CRP c-reactive protein, HB hemoglobin, WBC white blood cell, PLT platelet, RF rheumatoid factor, CCP cyclic citrullinated peptide antibody, IPF interstitial pulmonary fibrosis.

3.1 The methylation level of LDLRAD4 gene in RA and control group

The total methylation level of LDLRAD4 gene in RA group was significantly lower than that in control group (GroupDiff-0.0492714, $p < 0.001$, Fig. 2A). Four gene regions showed the same resultes, especially LDLRAD4-43F and LDLRAD4-44F (Fig. 2B-E).

A total of 89 CpG sites in 4 gene regions (31 in LDLRAD4-42-1F, 14 in LDLRAD4-42-2F, 20 in LDLRAD4-43F, and 24 in LDLRAD4-44F) were sequenced in this study. Statistically significant differences were found in 49 CpG sites compared with the control group, and methylation levels of all 49 CpG sites in RA group were lower. Among them, there were 2 in LDLRAD4-42-1F, 3 in LDLRAD4-42-2F, 20 in LDLRAD4-43F, and 24 in LDLRAD4-44F, mainly concentrated in LDLRAD4-43F and LDLRAD4-44F regions. The group difference ranged from − 0.0023896 to -0.1164491.

The methylation levels of LDLRAD4 gene were analyzed with clinical characteristics. Age, BMI, gender, hypertension, D-dimer, PLT, ESR, CRP showed weak correlation with the methylation levels of certain gene regions and CpG sites, as well as anti-CCP values. The absolute values of these correlation coefficients ranged from 0.140 to 0.370. No relationship was found between methylation level and RF values.

3.2 Predictive Model and ROC curves for RA

The total methylation level of LDLRAD4 gene and the methylation levels of each region were applied to predict RA and plot ROC curves (Fig. 2F and Table 2), which showed that the AUC values were from 0.64 to 0.78, the sensitivity was from 0.67 to 0.70, and the specificity was from 0.57 to 0.80. It can be seen that the methylation levels of LDLRAD4 gene have certain specificity and sensitivity for the diagnosis of RA, especially LDLRAD4-44F region, whose AUC value was 0.78.

Table 2
The sensitivity/specificity for predicting RA

Gene regions	Sensitivity	Specificity	Accuracy	AUC
LDLRAD4	0.69	0.74	0.72	0.77
LDLRAD4-42-1F	0.68	0.58	0.63	0.65
LDLRAD4-42-2F	0.67	0.57	0.62	0.64
LDLRAD4-43F	0.70	0.65	0.68	0.71
LDLRAD4-44F	0.69	0.80	0.74	0.78

3.3 The relationship between methylation levels and inflammation indicators in RA group

The study showed weak correlation between methylation levels and ESR, CRP in RA group. To further evaluate the relationship between methylation levels and inflammation indicators, the RA group was divided into four groups according to RF and anti-CCP values: RF+/CCP+, RF+/CCP-, RF-/CCP + and RF-/CCP- group. Age, course, ESR, CRP, DAS28, leukocyte, hemoglobin, platelet, D-dimer, blood uric acid, LDLRAD4 methylation levels of the 4 groups were compared, and no significant statistical difference was found.

Correlation analysis between methylation levels and inflammation indicators was conducted in these four groups (Table 3). It was found that: in RF+/ CCP-group, the methylation levels of LDLRAD4, LDLRAD4-42-1F, LDLRAD4-43F and LDLRAD4-44F were strongly correlated with inflammatory indicators (DAS28, ESR and CRP), and the absolute value of r ranged from 0.566 to 0.767; in RF-/CCP + group, the methylation level of LDLRAD4-42-2F were strongly correlated with ESR and CRP, and the absolute value of r ranged from 0.638 and 0.693, respectively. This was also confirmed in CpG sites in these regions. In RF+/ CCP-group, part CpG sites in LDLRAD4-42-1F, LDLRAD4-43F and LDLRAD4-44F were strongly

correlated with inflammatory indicators DAS28, ESR and CRP, especially in LDLRAD4-43F and LDLRAD4-44F regions (Schedule 2). In RF-/CCP + group, part CpG sites in LDLRAD42-2 region were strongly correlated with ESR and CRP (Schedule 3).

Table 3
The correlation between methylation levels and DAS28, ESR, CRP in four RA groups

		LDLRAD4				
			LDLRAD4-42-1F	LDLRAD4-42-2F	LDLRAD4-43F	LDLRAD4-44F
RF+/CCP+	DAS28	-0.042	-0.143	-0.021	-0.115	-0.082
(n = 96)	ESR	-0.045	-0.023	0.05	-0.041	-0.016
	CRP	-0.105	-0.079	-0.027	-0.124	-0.101
RF+/CCP-	DAS28	− .597*	− .722**	-0.281	− .616*	− .580*
(n = 13)	ESR	− .597*	− .767**	-0.022	− .688**	− .619*
	CRP	-0.538	− .671*	-0.269	− .632*	− .566*
RF-/CCP+	DAS28	-0.288	-0.016	-0.426	-0.197	-0.309
(n = 16)	ESR	-0.176	-0.033	− .638**	-0.256	-0.244
	CRP	-0.311	-0.413	− .693**	-0.382	-0.371
RF-/CCP-	DAS28	-0.058	-0.036	-0.318	-0.085	-0.088
(n = 25)	ESR	-0.232	-0.09	− .432*	-0.275	-0.24
	CRP	-0.301	0.021	-0.348	-0.345	-0.268
*p < 0.05						
** p < 0.01						

4. Discussion

This research investigated LDLRAD4 methylation relationship in Chinese RA population, and hypomethylation of LDLRAD4 gene was observed. RA is an autoimmune disease characterized by erosive arthritis and multiple organs involved. The pathogenesis is not completely clear, and is related to genetic, environmental and other factors. Epigenetics is sensitive to external stimuli and mediates gene-environment interactions. Three epigenetic mechanisms have been described, including non-coding RNA, histone modification, and DNA methylation[2]. DNA methylation is the most widely studied mechanism in epigenetic inheritance. On the one hand, DNA methylation plays an important role in the course of RA and can be used as a target for treatment and diagnosis[8, 9]. On the other hand, DNA

methylation can be used as an observational indicator after the treatment of RA to providing new evidence for the use of DMARDs drugs and biologics（2, 5）.

LDLRAD4, also known as C18orf1, is located on chromosome 18 and expressed in multiple organs, including immune organs (lymph nodes, spleen). It was initially considered to be a susceptibility gene of schizophrenia, and its dysfunction may destroy the balance of excitatory/inhibitory transmission in the hippocampus, leading to the occurrence of schizophrenia(6). A number of later studies have confirmed that LDLRAD4 is highly expressed in various malignant tumors such as liver cancer, breast cancer, stomach cancer and colon cancer, and plays a positive role in promoting the proliferation and migration of cancer cells. High expression of LDLRAD4 is a risk factor for poor prognosis of pancreatic adenocarcinoma and gastrointestinal stromal tumor（6, 10, 11）. LDLRAD4 is a negative regulator for TGF-β signal, and TGF-β is an important inflammation cytokine involved in the pathogenesis of RA. LDLRAD4 can prevent the propagation of intracellular signals by competing with SARA and interacting with SMAD2 and SMAD3（11）. It can also play a role in cell proliferation and migration through the interaction with E3 ubiquitin ligase Nedd4.

Our study found that the methylation levels of LDLRAD4 gene, four regions and CpG sites were lower in the RA group, especially in LDLRAD4-43F and 44F regions, suggesting that the DNA methylation of LDLRAD4 gene may be involved in the pathogenesis of RA. The methylation levels of LDLRAD4 gene are found to be of value in diagnosis of RA, especially the methylation level of LDLRAD4-44F region with an AUC value of 0.78. However, no strong correlation was found between the methylation levels and anti-CCP values or RF values. Between control group and RF-/CCP- RA group, no significant statistical difference was found in the methylation levels of LDLRAD4 gene and regions, suggesting that LDLRAD4 is not be a specific diagnostic indicator for RF-/CCP- RA.

The DNA methylation of different LDLRAD4 regions are associated with inflammatory activity in different RA groups. In RF+/ CCP-group, the methylation levels of LDLRAD4, LDLRAD4-42-1F, LDLRAD4-43F and LDLRAD4-44F were strongly correlated with inflammatory indicators, especially LDLRAD4-43F and LDLRAD4-44F regions; in RF-/CCP + group, the methylation level of LDLRAD4-42-2F was strongly correlated with ESR and CRP, as well as CpG sites in these regions. This suggested that: (1) The methylation levels of LDLRAD4-43F and LDLRAD4-44F regions can be used as an inflammatory and therapeutic index in RF+/CCP- RA, as well as LDLRAD4-42-2F in RF-/CCP + RA; (2) The pathological effect of LDLRAD4 gene is not exactly same in RF+/CCP- and RF-/CCP + RA groups. However, the number of the four RA groups is not equal, and the conclusion need more participants and molecular mechanisms research.

5. Conclusions

In summary, LDLRAD4 hypomethylation level is significant in patients with RA. DNA methylation of LDLRAD4 gene may play an important role in the pathogenesis of RA, and is a potential diagnostic and inflammatory indicators for RA.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

Approval to conduct this human subjects research was obtained by the Ethics Committee of Qilu Hospital of Shandong University(Qingdao). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all individual participants included in the study.

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Author Contribution

S.J. and L.Z. wrote the main manuscript text, Y.F. and Z.T. helped to conduct data analysis and prepared figures and tables, and P.Z. design topic. All authors reviewed the manuscript.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

References

1. SCOTT DL, WOLFE F, HUIZINGA TW (2010) Rheumatoid arthritis. Lancet (London England) 376:1094–1108
2. CIECHOMSKA M (2019) ROSZKOWSKI L, MASLINSKI W. DNA Methylation as a Future Therapeutic and Diagnostic Target in Rheumatoid Arthritis, vol 8. Cells
3. NAKANO K, WHITAKER JW, BOYLE DL, WANG W, FIRESTEIN GS (2013) DNA methylome signature in rheumatoid arthritis. Ann Rheum Dis 72:110–117

4. LIU CC, FANG TJ, OU TT, WU CC, LI RN, LIN YC et al (2011) Global DNA methylation, DNMT1, and MBD2 in patients with rheumatoid arthritis. Immunol Lett 135:96–99

5. DE ANDRES MC, PEREZ-PAMPIN E, CALAZA M, SANTACLARA FJ, ORTEA I, GOMEZ-REINO JJ et al (2015) Assessment of global DNA methylation in peripheral blood cell subpopulations of early rheumatoid arthritis before and after methotrexate. Arthritis Res therapy 17:233

6. LIU Z, HUO X, ZHAO S, YANG J, SHI W (2017) Low density lipoprotein receptor class A domain containing 4 (LDLRAD4) promotes tumorigenesis of hepatic cancer cells. Exp Cell Res 360:189–198

7. ALETAHA D, NEOGI T, SILMAN AJ, FUNOVITS J, FELSON DT, BINGHAM CO et al (2010) 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthr Rhuem 62:2569–2581

8. ZHAO J, XU L, CHANG C, JIANG P, WEI K, SHI Y et al (2022) Circulating methylation level of HTR2A is associated with inflammation and disease activity in rheumatoid arthritis. Front Immunol 13:1054451

9. JIANG P, WEI K, XU L, CHANG C, ZHANG R, ZHAO J et al (2022) DNA methylation change of HIPK3 in Chinese rheumatoid arthritis and its effect on inflammation. Front Immunol 13:1087279

10. ITO Y, NAKAJIMA K, MASUBUCHI Y, KIKUCHI S, SAITO F, AKAHORI Y et al (2019) Expression Characteristics of Genes Hypermethylated and Downregulated in Rat Liver Specific to Nongenotoxic Hepatocarcinogens. Toxicol Sci 169:122–136

11. XIE W, XIAO H, LUO J, ZHAO L, JIN F, MA J et al (2020) Identification of low-density lipoprotein receptor class A domain containing 4 (LDLRAD4) as a prognostic indicator in primary gastrointestinal stromal tumors. Curr Probl Cancer 44:100593