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Letter to the Editors-in-Chief

Alternated mRNA expression of the genes in chromosome 9p21 is associated with coronary heart disease and genetic variants in chromosome 9p21

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1. Introduction

There is no doubt that genetic factor plays an important role in the onset of coronary heart disease (CHD). Study firstly reported that there were some CHD risk sites in chromosome 9p21 region in 2007 [1]. And the results were verified in different populations by scholars from different countries. But the potential mechanisms of genetic variants in chromosome 9p21 effects on the onset and development of CHD is still unclear.

Chromosome 9p21 region is characterized by the feature of a high degree of linkage disequilibrium. The single nucleotide polymorphisms (SNPs) related to CHD in chromosome 9p21 region are adjacent to some functional genes, such as *CDKN2A*, *CDKN2B*, *ANRIL* and *MTAP*. Considering that, we hypothesized that these SNPs may be linked to the adjacent genes and affect the transcription of the genes. The present study was conducted to explore the relationships among the reported CHD risk SNPs, the relative mRNA expression levels of *CDKN2A*, *CDKN2B*, *ANRIL* and *MTAP* and CHD.

2. Materials and methods

2.1. Subjects

A total of 583 unrelated patients with CHD from the First Hospital of Jilin University, Changchun, China, were recruited between 2009 and 2012. All patients had been examined by standardized coronary angiography, and diagnosed with CHD based on one or more major coronary arteries with 50% or greater stenosis. Among the 583 patients, 91 patients were diagnosed with CHD for the first time, and had not used any drugs to treat CHD.

Comparable control subjects ($n = 540$) were randomly selected from the same hospital in the routine check-up as part of annual body examination, including an electrocardiogram (ECG), chest X-ray and serum analysis. They were classified as healthy subjects based on their physical examination coupled with the absence of personal or family history and other reasons to suspect CAD. Individuals were excluded from having congenital heart disease, cardiomyopathy, liver and renal disease.

All the subjects were given an informed consent and were well told of the study protocol. The study was approved by the ethics committee of school of public health, Jilin University, Changchun, China. Peripheral venous blood was collected in tubes containing disodium-EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for DNA. Fresh peripheral venous blood was collected for mRNA and protein extraction.

2.2. Genotyping

Five reported coronary heart disease risk locus of Chinese Han population (rs2383206, rs2383207, rs10757274, rs10757278 and rs1333049) in chromosome 9p21 region were selected as genetic markers.

Genomic DNA used for SNPs genotyping was extracted from 583 CHD patients' and 540 controls' peripheral blood leukocytes using a DNA extraction kit (TianGen, Beijing, China). SNPs were genotyped using SEQUENOM Mass-ARRAY system with amplification primers and extension primers described in Supplementary Table 1.

2.3. Real-time fluorescence quantitative PCR

In order to control the influence of medicines on the read-outs, only the 91 patients were selected to detect the expression levels of mRNA from the 583 CHD patients. Based on the basic characteristics of the 91 CHD patients, we selected 30 comparable controls from the 540 controls.

Total RNAs were isolated from the leukocytes in fresh blood using Trizol reagent (TaKaRa Bio Group, Shiga, Japan). Real-time fluorescence quantitative PCR (RT-PCR) was used to detect the mRNA levels of *CDKN2A*, *CDKN2B*, *MTAP* and *ANRIL*. Beta-actin was used as an internal control. The mRNA levels were quantified via $2^{-\Delta CT}$. The primers are listed in Supplementary Table 2.

2.4. Statistical analysis

Based on the normality, the data were presented as the mean ± standard deviation or median (quartile range), and analyzed with *t*-test, one-way ANOVA or Wilcoxon rank sum test using SPSS 16.0 program.

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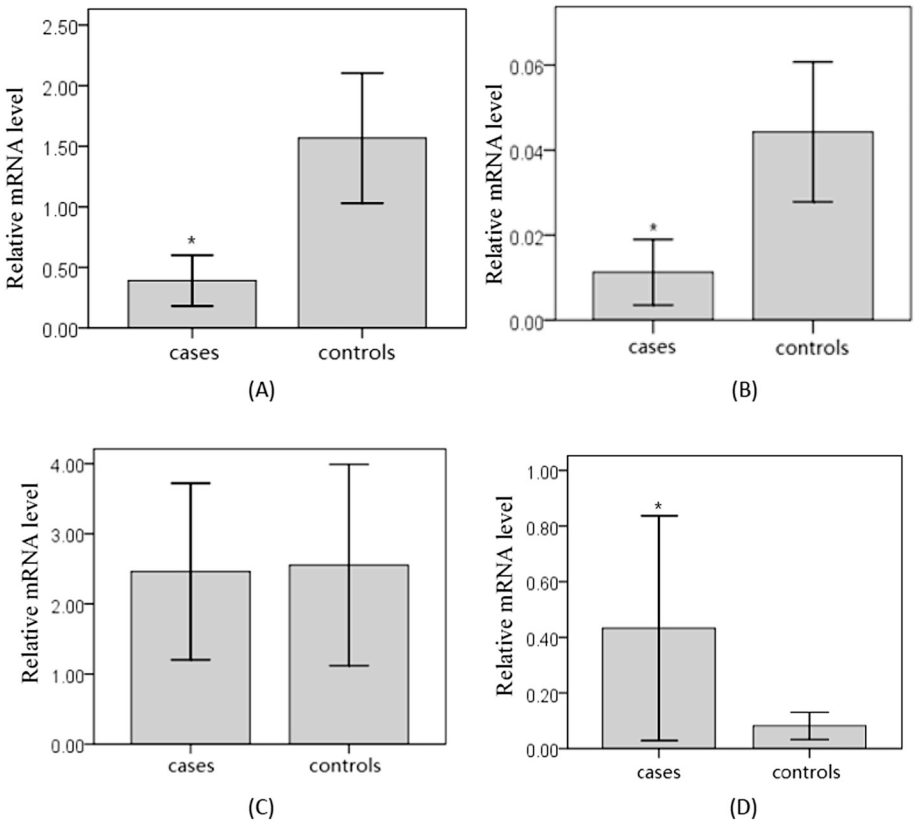

Fig. 1. The mRNA levels of target genes in cases and controls
(A) The relative mRNA levels of *CDKN2A*; (B) The relative mRNA levels of *CDKN2B*; (C) The relative mRNA levels of *ANRIL*; (D) The relative mRNA levels of *MTAP*. $^{*}P < 0.05$, compared with controls.

3. Results and discussion

There were no significant differences of the mean age, sex and body mass index (BMI) between the CHD patients and the controls. (Supplementary Table 3).

Previous studies had proved that genetic variants in chromosome 9p21 region were contributed to CHD. In this study, five reported coronary heart disease risk locus (rs2383206, rs2383207, rs10757274, rs10757278 and rs1333049) in chromosome 9p21 region were selected to study the relationship. The genotypic distributions of 5 SNPs were not deviated from Hardy–Weinberg equilibrium in both case and control groups. And we confirmed that the rs2383207, rs10757274, rs10757278, rs1333049, and rs2383206 loci in chromosome 9p21 are susceptible loci for CHD in northern Chinese Han population; the C allele of rs1333049 and the G alleles of the other 4 SNPs were the risk alleles of CHD (Supplementary Table 4).

Atherosclerosis, an inflammatory disease characterized by plaque formation, is the pathological basis of CHD. Previous studies indicated that leukocytes in peripheral blood played an important role in plaque formation and progression [2], and the mRNA expression levels of some genes in peripheral blood leukocytes are related to CHD [3]. In this study, we detected the mRNA expression levels of the *CDKN2A*, *CDKN2B*, *MTAP* and *ANRIL* in the peripheral blood leukocytes of subjects.

The *CDKN2A* and *CDKN2B* are mainly involved in cell proliferation and apoptosis. VinuÉ Á et al. found that changes in CDKN2A/2B expression were associated with T-cell phenotype modulation [4]. All of them are important features of atherosclerosis. Study indicated that the *CDKN2A* and *CDKN2B* may be related to pathogenesis of atherosclerosis [5]. Our results demonstrated that the mRNA expression levels of *CDKN2A* and *CDKN2B* in the peripheral blood leukocytes of patients with CHD were significantly lower than those in the controls (Fig. 1). It was probable that the phenomenon of under-regulation of *CDKN2A* and *CDKN2B* genes expression may occur in the process of the development of CHD, what was a common phenomenon of the process of destruction of normal tissues and cells.

MTAP gene encodes an enzyme that plays a major role in polyamine metabolism and is important for the salvage of both adenine and methionine. The relationship between *MTAP* and atherosclerosis is inconsistent [6,7]. Our results showed that the mRNA level of *MTAP* in patients with CHD were significantly higher than those in the control group (Fig. 1), suggesting that the increased mRNA expression level of *MTAP* gene may be related to incidence of CHD in the northern Chinese Han population. As CHD is a chronic progressive disease, the over-regulation of MTAP expression also accelerates the occurrence and progress of CHD.

ANRIL gene is located within the *CDKN2B-CDKN2A* gene cluster at chromosome 9p21 and was expressed in atherosclerotic affected tissues and cells. *ANRIL* gene product is a functional RNA molecule that interacts with polycomb repressive complex-1 and -2, leading to epigenetic silencing of other genes in this cluster. In this study, there was no significant difference in the expression levels of *ANRIL* between the two groups (Fig. 1), indicating that the expression level of *ANRIL* may not be related to the occurrence of CHD in Chinese Han population. Gan Y et al. found that the expression of the genes in 9p21, and specifically *ANRIL* and *CDKN2A*, are coordinated in patients with colon carcinoma [8]. But in our study, the results showed the lack of correlation of ANRIL and *CDKN2A/2B* in patients with CHD, which is the same as the findings of Holdt et al. [9].

Then, according to different genotypes, we divided CHD patients into different groups to analyze the differences of *CDKN2A*, *CDKN2B*, *ANRIL* and *MTAP* gene expression levels in CHD patients and to explore the possible mechanism of genetic variations in chromosome 9p21 in CHD. The results showed that the mRNA expression levels of *CDKN2A* in peripheral blood of patients with different genotypes of rs2383207 were significantly different, while the expression levels of *CDKN2B*, *MTAP* and *ANRIL* were not (Table 1), indicating that rs2383207 may affect the

Table 1
The relationship between genetic variants and mRNA expression levels of target genes in patients.

SNPs		*CDKN2A*	*CDKN2B*	*MTAP*	*ANRIL*
rs1333049	GG(n = 25)	$3.043 \times 10^{-3}(6.826 \times 10^{-3})$	0.200(0.404)	$7.381 \times 10^{-2}(13.936 \times 10^{-2})$	1.292(3.248)
	CG(n = 49)	$4.842 \times 10^{-3}(15.465 \times 10^{-3})$	0.275(0.445)	$13.584 \times 10^{-2}(24.325 \times 10^{-2})$	1.526(2.116)
	CC(n = 16)	$5.346 \times 10^{-3}(15.869 \times 10^{-3})$	0.298(0.568)	$15.722 \times 10^{-2}(36.937 \times 10^{-2})$	1.481(3.111)
	χ^2	2.868	0.992	2.886	0.107
	P	0.238	0.609	0.236	0.948
rs2383206	AA(n = 29)	$3.001 \times 10^{-3}(7.084 \times 10^{-3})$	0.193(0.350)	$7.856 \times 10^{-2}(13.254 \times 10^{-2})$	1.292(3.027)
	AG(n = 46)	$4.842 \times 10^{-3}(13.525 \times 10^{-3})$	0.274(0.458)	$11.392 \times 10^{-2}(24.024 \times 10^{-2})$	1.446(2.178)
	GG(n = 16)	$5.679 \times 10^{-3}(16.773 \times 10^{-3})$	0.325(0.532)	$15.072 \times 10^{-2}(41.816 \times 10^{-2})$	1.481(3.541)
	χ^2	4.333	3.331	3.593	0.183
	P	0.115	0.189	0.166	0.913
rs2383207	AA(n = 29)	$1.271 \times 10^{-3}(2.225 \times 10^{-3})$	0.195(0.431)	$4.844 \times 10^{-2}(6.809 \times 10^{-2})$	2.003(6.772)
	AG(n = 31)	$4.425 \times 10^{-3}(7.534 \times 10^{-3})$	0.200(0.400)	$11.034 \times 10^{-2}(24.279 \times 10^{-2})$	1.537(2.034)
	GG(n = 31)	$5.411 \times 10^{-3}(11.248 \times 10^{-3})$	0.279(0.465)	$14.441 \times 10^{-2}(34.646 \times 10^{-2})$	1.292(3.128)
	χ^2	7.138	0.898	4.189	0.133
	P	0.028	0.638	0.123	0.936
rs10757274	AA(n = 28)	$3.012 \times 10^{-3}(7.954 \times 10^{-3})$	0.197(0.399)	$8.226 \times 10^{-2}(13.301 \times 10^{-2})$	0.922(2.820)
	AG(n = 48)	$4.842 \times 10^{-3}(11.296 \times 10^{-3})$	0.274(0.451)	$12.158 \times 10^{-2}(25.133 \times 10^{-2})$	1.564(3.209)
	GG(n = 15)	$5.489 \times 10^{-3}(20.191 \times 10^{-3})$	0.319(0.567)	$14.968 \times 10^{-2}(36.985 \times 10^{-2})$	1.292(1.800)
	χ^2	4.159	2.024	1.926	0.676
	P	0.125	0.364	0.382	0.713
rs10757278	AA(n = 27)	$3.043 \times 10^{-3}(5.438 \times 10^{-3})$	0.200(0.405)	$7.588 \times 10^{-2}(12.784 \times 10^{-2})$	1.292(3.122)
	AG(n = 46)	$5.299 \times 10^{-3}(15.552 \times 10^{-3})$	0.274(0.451)	$12.308 \times 10^{-2}(24.007 \times 10^{-2})$	1.531(2.392)
	GG(n = 18)	$5.013 \times 10^{-3}(13.922 \times 10^{-3})$	0.291(0.513)	$15.722 \times 10^{-2}(38.482 \times 10^{-2})$	1.067(2.274)
	χ^2	4.176	1.479	3.255	0.178
	P	0.124	0.477	0.196	0.915

The data were presented as median (quartile range), and the results were analyzed by Wilcoxon rank sum test.

occurrence of CHD by affecting the mRNA expression of *CDKN2A*. Other scholars had found that variants at 9p21.3 could regulate the expression levels of *CDKN2A* and *CDKN2B* by modulating *ANRIL* expression and/or structure [10]. There was no significant difference in the expression of *CDKN2A*, *CDKN2B*, *MTAP* and *ANRIL* in the peripheral blood of patients with different genotypes in the other four SNPs sites (Table 1), which indicated that the polymorphism of these sites do not affect the occurrence of CHD by affecting the expression of *CDKN2A*, *CDKN2B*, *MTAP*, *ANRIL* mRNA in peripheral blood. The mechanism of these genetic variations affecting CHD requires additional research to be elaborated.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.03.020.

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