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Original article

A common polymorphism in CD40 Kozak sequence (-1C/T) is associated with acute coronary syndrome

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ABSTRACT

Objective. – Evidence suggests the CD40-CD40L pathway as a key process in the development, progression, and outcome of acute coronary syndrome (ACS). We hypothesized that the -1C/T polymorphism of the CD40 gene would be associated with ACS and influence the CD40 expression.
Methods. – The genotype distribution and allele frequency of CD40-1C/T polymorphism in 248 ACS patients and 206 controls were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Monocytes from 90 healthy volunteers were incubated with IFN-γ. CD40 expression was detected by flow cytometry.
Result. – Patients with ACS showed a significant increase of CD40 expression compared with controls ($P < 0.001$). The frequency of the CC genotype in the ACS group was significantly higher than that of the controls ($P < 0.001$). Frequency of the C allele was higher among ACS patients compared with controls ($P < 0.001$). Case control association analysis of the CD40 -1C/T SNP showed an association between the C allele and ACS (OR = 1.991, 95%CI: 1.526 ~ 2.596, $P < 0.001$). -1C/C carriers presented significantly higher CD40 expression levels than -1C/T and -1T/T subjects, both in ACS group and controls ($P < 0.001$). When stimulated by IFN-γ, CD40 expression levels on monocytes in individuals with CC, CT and TT genotypes were increased by 9.16, 3.83 and 1.53 fold, respectively, compared with the levels absent with IFN-γ.
Conclusions. – These results suggest that the -1C allele of the CD40 (-1C/T) gene polymorphism is a genetic factor that may determine an individual's susceptibility by ACS in Chinese. The CD40 -1C/T polymorphism is a novel regulator of CD40 expression.

1. Introduction

Acute coronary syndrome (ACS) is currently considered the result of atherosclerotic plaques' disruption. Several studies clearly demonstrate that CD40 is a major trigger, eliciting a proinflammatory reaction in the vasculature, and plays an important role in the formation of atherosclerotic lesion [1]. This can be evidenced by the finding that inhibition of CD40/CD40L interaction reduces atherosclerosis in mice [2,3]. Recent studies strongly suggest that in the "shoulder" regions of lipid-rich plaques, which are known to contain dense inflammatory infiltrates, CD40 are positively expressed. Patients with ACS showed a significant increase of CD40 and CD40L coexpression on platelets compared with control and SA group [4]. These dates prove that the CD40-CD40L system plays a crucial role in the pathomechanism of ACS. It has been proved that a C/T polymorphism in the Kozak sequence of the CD40 gene enhances the efficiency of CD40 gene translation [5]. However, the relationship between the polymorphism and ACS and whether the C/T single nuclear polymorphism could influence the CD40 expression on B cells remain little reported. Therefore, we established a case-control study to analyse the association of ACS and C/T polymorphism in the CD40 gene, as well as the CD40 expression on B cells and monocytes.

2. Patients and methods

2.1. Patients and controls

2.1.1. Patients with ACS

Two hundred and forty eight patients with ACS were collected, which includes Q-wave myocardial infarction, non Q-wave myocardial infarction or unstable angina. Q-wave myocardial infarction was defined by the presence of (1) chest pain typical for myocardial ischemia lasting > 30 min, (2) elevation of total creatine kinase to at least twice the upper limit of normal with an

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elevated creatine kinase-MB fraction, and (3) development of diagnostic Q waves in ≥ 2 electrically contiguous leads. Non-Q-wave myocardial infarction was defined in the same manner, but without the appearance of new Q waves. Unstable angina was defined in a standard fashion. Coronary angiography was performed in multiple right and left anterior oblique projections with cranial and caudal angulation for visualization of all segments of the coronary arteries. Coronary artery disease was considered present if there was a stenosis diameter narrowing ≥ 50% in at least one of three major epicardial coronary arteries.

2.1.2. Control

Two hundred and six age- and gender-matched persons with no personal or family history of coronary heart disease and no stenosis in coronary artery (coronary angiography was performed) served as controls in our association studies. Chronic inflammatory disease, autoimmune disease and carcinoma were excluded. All the patients and controls reside in Hubei province, China. The study plan was reviewed and approved by the institutional review committee, and informed consent was obtained from all patients and control subjects.

2.2. Genotyping

Genomic DNA was extracted from whole blood with the methods previously described [6]. The C/T polymorphism at position -1 of CD40 gene was amplified by polymerase chain reaction(PCR) from genomic DNA using following primers [7] 5′-CCT CTT CCC CGA AGT CTT CC-3′, and 5′-GAA ACT CCT GCG CGG TGA AT-3′ (GenBank accession number AL035662). The reaction was carried out with 100 ng of genomic DNA in a volume of 25 μl containing 6 pmol of each primer, 1.5 mmol/L Mg^{2+}, 200 μmol/L of each dNTP and 1.0 U of Taq DNA polymerase. After predenaturation at 95 °C for 5 min, DNA fragments were amplified for 35 cycles by the following steps: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 10 min. All PCR reactions were performed in a GeneAmp PCR System 2700, USA. The DNA products were digested with the restriction enzyme *Nco*I (New England Biolabs, Beverly, USA). The digests were analyzed on 2% agarose gel electrophoresis. PCR product from patients with each representative genotype was sequenced using an automated DNA sequence analyzer (PRISM 373A, Applied Biosystems) and confirmed that the sequences corresponded to the genotypes obtained from restriction fragment length polymorphism.

2.3. Cell culture

We selected 90 healthy blood donors, 30 with -1C/C genotype, 30 -1C/T genotype and 30 -1T/T genotype, matched for age and sex. Peripheral blood mononuclear cells were separated by density gradient centrifugation on a Ficoll-Hypaque discontinuous gradient. They were cultured in RPMI 1640 with 10% heat-inactivated FBS serum and cultured in flat-bottom plats. The nonadherent peripheral blood monoclear cell fraction was separated after 1 h by gentle washes with RPMI 1640 medium. Adherent cells were subsequently removed by rapid flushing with PBS at 4 °C, centrifuged, and cultured with RPMI 1640 medium at a density of 1×10^6 cells per well. In general, cells were incubated in the absence or the presence of IFN-γ (100 ng/ml) for 24 h. The CD40 expression on extrasomatic cultural mononuclear cell was analysed by flow cytometry following the manufacturer's instructions.

2.4. Flow cytometry

100 μl whole blood was incubated (1 h, 4 °C) with PE-conjugated mouse anti-human CD19 and FITC-conjugated mouse anti-human CD40 or FITC-conjugated control IgG. Subsequently, the cells were hemolysis and analyzed in a Coulter, Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany). Monocytes were washed with ice-cold PBS, harvested by trypsinization, and fixed. Subsequently, the cells were washed once with PBS/2% BSA before being incubated (1 h, 4 °C) with either buffer alone or FITC-conjugated control IgG, mouse anti-human CD40. Finally, cells were washed with PBS/2% BSA and analyzed in flow cytometer. Date are expressed as mean fluorescence intensity.

2.5. Statistical analysis

Descriptive results of continuous variables are expressed as mean ± SE. Before statistical analysis, normal distribution and homogeneity of the variances were tested. Parameters that did not fulfil these test (Lpa) were log-transformed. We used χ^2 test for comparison of proportions. Comparison of variables between groups of subjects was performed using Student's *t* test or one-way ANOVA. Levels of statistical significance were set at $P < 0.05$.

3. Results

3.1. Clinical and laboratory characteristics in the patients and controls

The characteristics of the patient and control groups are shown in Table 1. The ACS patients are found to be more likely to have hypertension and diabetes. Lipid profiles revealed that the ACS patient group have a significantly lower mean level of HDL-cholesterol and apolipoprotein AI ($P < 0.01$). SBP, DBP and Lp(a) are significantly higher in the ACS patients than that in the controls. Sex, age, BMI, FBG, total cholesterol, triglycerides, LDL- cholesterol, and apolipoprotein B levels are not significantly different between the patient and control groups.

3.2. Distribution of allele and genotype frequencies

The distribution of the genotypes and alleles is shown in Table 2, and they are all consistent with Hardy-Weinberg equilibrium. There are significant differences in frequencies of allele and genotype in -1C/T polymorphism between ACS and

Table 1
The characteristics of the patient and control groups.

Groups	Control (n = 206)	ACS (n = 248)	P-value
Sex (M/F)	136/70	158/90	NS
Age (Y)	59.8 ± 9	63.8 ± 10	NS
BMI (kg/m^2)	24.1 ± 2.8	25.2 ± 2.9	NS
Hypertension (yes/no)	55/151	135/113	< 0.01
Diabetes (yes/no)	48/158	117/131	< 0.01
SBP (mmHg)	122.6 ± 15.5	141.2 ± 27.2	< 0.01
DBP (mmHg)	76.7 ± 8.4	85.8 ± 20.4	< 0.01
FBG (mmol/L)	5.4 ± 0.8	5.8 ± 1.8	NS
CHOL (mmol/L)	4.9 ± 1.1	4.6 ± 1.0	NS
TG (mmol/L)	1.6 ± 1.7	1.7 ± 1.3	NS
HDL- C (mmol/L)	1.5 ± 0.3	0.9 ± 0.3	< 0.01
LDL-C (mmol/L)	3.0 ± 0.8	2.9 ± 0.9	NS
lgLp(a)[a]	4.3 ± 0.8	5.2 ± 1.1	< 0.01
Apolipoprotein AI (g/L)	1.2 ± 0.3	0.8 ± 0.2	< 0.01
Apolipoprotein B (g/L)	0.8 ± 0.2	0.8 ± 0.3	NS
CD40 (MFI)	55.1 ± 15.9	72.8 ± 18.2	< 0.001

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting serum glucose; CHOL: Total cholesterol; TG: Triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.
[a] Lp(a) did not fulfil normal distribution and was log-transformed.