

Value analysis of CD69 combined with EGR1 in the diagnosis of coronary heart disease

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Abstract. Expression and clinical significance of CD69 and early growth response (EGR1) in plasma of patients with coronary heart disease (CHD) were investigated. A total of 194 patients with CHD and 130 healthy subjects, respectively, were selected as CHD and control group, clinical data were collected and coronary angiography was performed. RT-qPCR was used to detect the expression of EGR1. Flow cytometry was used to detect the expression level of CD69 and the receiver operating characteristic curve was used to analyze the values of relative expression of CD69 and EGR1. The relative expression of CD69 in plasma of patients with CHD was higher than that in control group, while the relative expression of EGR1 was lower than that in control group. The relative expression of EGR1 in plasma of patients with CHD was negatively correlated with lipoprotein a [Lp(a)] and high sensitive C-reactive protein (hs-CRP) ($r=-0.394$ and -0.524 , $P<0.05$), and the relative expression of CD69 in peripheral blood was positively correlated with [Lp(a)] and hs-CRP ($r=0.352$ and 0.402 , $P<0.05$). The area under curve (AUC) of the relative expression of CD69 in peripheral blood of patients with CHD in evaluating the course of the disease of patients was 0.889 (95% CI: 0.822-0.958). The AUC of the relative expression of EGR1 in plasma in evaluating the course of the disease of patients was 0.933 (95% CI: 0.867-0.978). By the combined detection of CD69 and EGR1, it was found that the AUC was 0.954 (95% CI: 0.887-0.982). The expression level of EGR1 in plasma of patients with CHD decreased, while the expression level of CD69 increased, and both of them were related to the severity of the disease of patients, which could be used as an indicator to evaluate the progression of the patients' conditions.

Introduction

Coronary heart disease (CHD), usually refers to the heart disease caused by myocardial ischemia and hypoxia due to

insufficiency of myocardial blood supply in patients, which is caused by stenosis and obstruction of blood vessel lumen as a result of coronary atherosclerosis (AS) (1,2). AS is mainly caused by the participation of lipid accumulation in the wall of coronary artery and inflammation and immune response (3). The data released by the Statistical Commission of the American Heart Association in 2009 showed (4) that cardiovascular disease has become the top ranking disease in the causes of human death in the world. In China, CHD has become one of the two leading causes of death after malignant tumor, for China has gradually entered an aging society, and the number of elderly people has gradually increased (5). Another study finds (6) that the main risk factors for CHD are age, sex, smoking and diabetes mellitus, and other secondary factors are diet, family history, exercise volume and congenital defects.

CD69 is the earliest expressed signal transduction molecule on the surface of immune system activated T lymphocytes (7). There is a study showing (8) that the downregulation of the expression of CD69 can decrease the activation of CD4⁺T cells in I diabetic mouse model, thus regulating the immune response to the disease. Early growth response (EGR) is an early gene and its family includes four members: *EGR1*, *EGR2*, *EGR3* and *EGR4* (9). EGR can be activated by a variety of extracellular signal molecules to cause cascade amplification of cells, and all genes in the family contain a highly conserved structure domain that binds to DNA. This domain consists of three same zinc finger structures (Cys₂ His₂), which can bind specifically to the downstream promoter region and play a regulatory and transcriptional role (10). *EGR1* gene is more studied in EGR family and many studies have reported that EGR1 is directly or indirectly involved in the proliferation and differentiation of tumor cells (11). However, CD69 and EGR1 are rarely reported in CHD. We found that EGR1 and CD69 were differentially expressed in CHD by screening from ceo database. Therefore, in this study, the clinical effects of EGR1 and CD69 in CHD were investigated through the detection of the expression of EGR1 and CD69 in the blood of patients with CHD.

Patients and methods

General data of patients. In the present study, 194 patients with CHD, admitted to the Department of Cardiology in People's Hospital of Hunan Province (Changsha, China)

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from June 2015 to September 2016, were selected as experimental group. Another 130 normal volunteers at the same time in the physical examination center were selected as control group. There were 112 males and 82 females in experimental group, aged from 39-78 years, with an average age of 66.5 ± 11.3 years. The patients in experimental group were diagnosed by coronary angiography and all the patients in experimental group were patients with CHD after the examination. There were 70 males and 60 females in control group, aged from 42-80 years, with an average age of 64.8 ± 10.9 years. Patients in control group were excluded from CHD and other systemic heart diseases. Biochemical indicators of clinical data of patients were collected, including total cholesterol (TC), triacylglycerol (TG), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C), lipoprotein a [Lp(a)], fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), high sensitive C-reactive protein (hs-CRP) and serum creatinine (Scr), and statistical analysis was performed.

This study was approved by the Medical Ethics Committee of People's Hospital of Hunan Province (Changsha, China) and patients and their family members were informed and signed the informed consent.

Inclusion and exclusion criteria. Inclusion criteria were: There were $\geq 50\%$ stenosis in one or more main coronary arteries according to coronary angiography, patient was >18 years and course of disease was half a year. Patient had no recent drug treatment, no other hereditary disease, no radiotherapy and chemotherapy, no autism, memory impairment and hearing impairment. Patient cooperated perfectly with follow-up and clinical information.

Exclusion criteria were: Malignant tumors, severe dysfunction of important organs, acute myocarditis and pericarditis, congenital heart disease, immune dysfunction, connective tissue disease, chronic infection, pulmonary embolism and cerebrovascular disease.

Main reagents and instruments. CD69 McAb (mouse anti-human-phycoerythrin labeled), CD3 McAb (mouse anti-human-activated protein), CD4 McAb (mouse anti-human-fluorescein isothiocyanate), PE-labeled mouse IgG1 and CD69 isomorph control were purchased from BD Biosciences (San Jose, CA, USA). Erythrocyte splitting liquor was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). TRIzol was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Reverse transcriptase and reverse transcription kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). 2X SYBR-Green qPCR mix and revert aid first strand cDNA synthesis kit were purchased from Invitrogen; Thermo Fisher Scientific Inc. ABI Prism 7900 PCR instrument was purchased from Thermo Fisher Scientific, Inc.

CD69 detection

Sample collection and processing. Venous whole blood (5 ml) was extracted with vacuum tubes (EDTA- Na_2) on an empty stomach and mixed with Hanks solution at 1:1. Then the mixture was superimposed on the liquid level of 8 ml lymphocyte separating and was centrifuged at $543 \times g$ for 15 min at

25°C . After centrifugation, lymphocytes on the second layer (annular milky white) were collected and put into test tube (containing 10 ml of Hanks solution). Then they were mixed and centrifuged at $543 \times g$ again for 15 min at 25°C . The supernatant was discarded, the precipitation was left, and cells were resuspended and washed. Finally, cell concentration was adjusted to $1 \times 10^6/\text{ml}$ by 10% RPMI-1640 culture medium, and resuspended suspension was added to a 24-orifice plate (1 ml per orifice). A total of 100 $\mu\text{l}/\text{ml}$ for penicillin, streptomycin (100 $\mu\text{l}/\text{ml}$) and PHA (20 $\mu\text{g}/\text{ml}$) for stimulator were added and mixed. The culture was performed (20 h at 37°C and 5% CO_2 incubator), the resuspension was performed by PBS, and the cell concentration was adjusted to $1 \times 10^6/\text{ml}$.

Sample detection. Two cell suspensions (100 μl) were added to the sample tube. One sample was added to each of the 10 μl of CD3, CD4 and CD69 McAbs, respectively, and the other one was added to the same type of CD3 McAb, CD4 McAb and CD69 McAb, respectively. Cell suspensions were incubated in greenhouse for 20 min and treated without light. After dyeing, 2 ml of erythrocyte splitting liquor was added for mixing. The cell suspensions were static for 10 min and dissociated without light, and the dissociated test tube was centrifuged at $1,006 \times g$ for 5 min at 25°C . The supernatant was discarded, and 2 ml of PBS was added to carry on resuspension of the cells. After centrifugation at $543 \times g$ for 15 min at 25°C , the untuberculous antibody was removed, the supernatant was discarded, and 0.5 ml of PBS was added for resuspension. Flow cytometry was used to detect the expression level of CD69 in $\text{CD}3^+\text{CD}4^+\text{T}$ cells.

EGR1 detection

Sample collection and processing. Venous whole blood (5 ml) was extracted with vacuum tubes (EDTA- Na_2) on an empty stomach and centrifuged at $1,006 \times g$ for 5 min at room temperature within 30 min after extraction. Supernatant was discarded, and plasma was transferred to EP tube and stored at -80°C in a refrigerator. Total RNA of frozen plasma was extracted with TRIzol reagent, and extraction procedure was carried out in accordance with the manufacturer's protocol. UV spectrophotometer (L6S; INESA Analytical Instrument Co. Ltd., Shanghai, China) was used to detect the concentration and purity of RNA (the A260/A280 of total RNA solution was within the range of 1.8-2.1), and quality of the total RNA was analyzed by 1% denatured agarose gel electrophoresis. Reverse transcription procedure was carried out strictly according to the instruction of reverse transcription kit.

RT-qPCR detection. Design and synthesis of EGR1 primers were carried out by Shanghai Bioengineering Co., Ltd., (Shanghai, China) (Table I). PCR reaction kit (2X SYBR-Green qPCR Mix) was used for the configuration of reaction system: A total of 0.2 μl for up- and downstream primers respectively, 1 μl for cDNA, 5 μl for SYBR Select Master Mix and double distilled water added to 10 μl ; ABI Prism 7900 PCR instrument was used for amplification. PrimeScript™ RT Master Mix (Takara Biotechnology Co., Ltd.) to reverse the total RNA collected according to the kit instructions. Reaction conditions: 2 min after pre-denaturation at 95°C , 15 sec for denaturation at 95°C , 60 sec for annealing at 60°C , 15 sec for extension

Table I. Primer sequence.

Genes	Upstream	Downstream
<i>EGR1</i>	5'-CCCTTGCTCCCTTCAATGCT-3'	5'-CGAAATCCATGGCACAGACAC-3'
<i>GAPDH</i>	5'-AGCCACATCGCTCAGACA-3'	5'-TGGACTCCACGACGTACT-3'

EGR1, early growth response.

at 95°C, 40 cycles. GAPDH was used as internal reference in this experiment, and was conducted three times. Results were analyzed by $2^{-\Delta C_q}$ method (12).

Statistical analysis. In the present study, SPSS 20.0 software package (IBM Corp., Armonk, NY, USA) was used for statistical analysis of the collected data, and GraphPad Prism 5 software (La Jolla, CA, USA) was used to plot the resulting data graph. In the present study, the measurement data are expressed by mean \pm standard deviation (mean \pm SD) and tested by t-test. The counting data was expressed by rate (%), and the comparison between groups was tested by χ^2 . In the present study, Pearson's correlation analysis was used to analyze the relationships among the variables, and ROC curve was used to plot the two indicators and to evaluate the values of the two indicators in the course of the disease of patients with CHD.

Results

Comparison of clinical treatment in patients. Through the comparison of the collected data of the two groups, it was found that there was no statistically significant difference in clinical data such as sex, age, smoking and alcohol and in biochemical indicators of TC, TG, LDL-C, FBG and HbA1c ($P > 0.05$). However, there were statistically significant differences in HDL-C, [Lp(a)], hs-CRP and Scr between patients in experimental and control group ($P < 0.05$), and expression level of HDL-C of patients in experimental was significantly lower than that in control group, while the expression level of [Lp(a)], hs-CRP and Scr in experimental was significantly higher than that in control group (Table II).

Expression of CD69 and EGR1 in both groups of patients. By comparing the expression of CD69 in two groups by flow cytometry, it was found that the expression of CD69 in experimental group was $16.58 \pm 4.39\%$, which increased significantly, compared with the control group $10.54 \pm 2.63\%$, and there was a significant difference in the expression of CD69 between the two groups, which was 1.57 times ($P < 0.05$). Through the RT-qPCR detection of the expression of EGR1 in plasma, it was found that the expression level of EGR1 (1.255 ± 0.362) in control group was significantly higher than that in experimental group (0.254 ± 0.094), and there was a significant difference between the two groups ($P < 0.05$) (Figs. 1 and 2).

Correlation analysis of the expression and clinical indicators of CD69 and EGR1 in patients with CHD. Through Pearson's correlation analysis, it was found that the expression level of CD69 in peripheral blood of patients with CHD was positively

Table II. Clinical information of patients (n).

Variables	Groups		t/χ^2	P-value
	Experimental (n=194)	Control (n=130)		
Sex			0.477	0.49
Male	112	70		
Female	82	60		
Age (years)			0.837	0.36
≥ 55	84	63		
< 55	110	67		
Smoking			0.003	0.954
Yes	120	80		
No	74	50		
Alcohol			0.338	0.561
Yes	51	38		
No	143	92		
TC (mmol/l)	1.77 ± 1.05	1.61 ± 0.72	1.411	0.159
TG (mmol/l)	4.55 ± 0.82	4.68 ± 0.94	1.317	0.189
HDL-C (mmol/l)	1.09 ± 0.22	1.42 ± 0.28	11.829	0.001
LDL-C (mmol/l)	2.34 ± 0.64	2.39 ± 0.71	0.659	0.511
[Lp(a)] (mg/l)	283.6 ± 66.5	235.8 ± 58.3	8.188	0.001
FBG (mmol/l)	5.84 ± 1.15	5.63 ± 0.89	1.757	0.08
HbA1c (%)	6.4 ± 1.0	6.2 ± 0.9	1.834	0.068
hs-CRP (mg/l)	5.64 ± 2.67	3.66 ± 2.31	6.894	0.001
Scr (μ mol/l)	83.5 ± 26.4	61.3 ± 17.6	8.409	0.001

TC, total cholesterol; TG, triacylglycerol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; [Lp(a)], lipoprotein a; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin; hs-CRP, high sensitive C-reactive protein; Scr, serum creatinine.

correlated with [Lp(a)] and hs-CRP, while the expression level of EGR1 in patients with CHD was negatively correlated with [Lp(a)] and hs-CRP, and the rest of the clinical indicator tables were not included due to no correlation (Table III).

Diagnostic values of the expression level of CD69 and EGR1 in patients with CHD. Through plotting the ROC curve of the data of the two groups, it was found that the AUC of the expression level of CD69 in peripheral blood was 0.889 (95% CI: 0.822-0.958), and the sensitivity was 73.4%, and the specificity was 86.5%. The higher the expression level

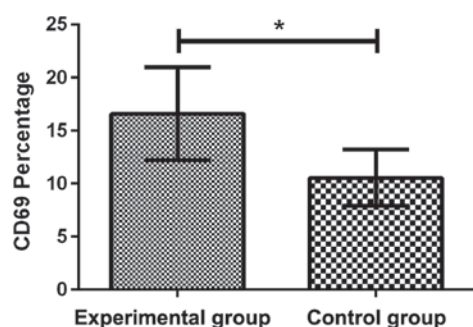


Figure 1. Expression of CD69 in both groups of patients. * $P<0.05$.

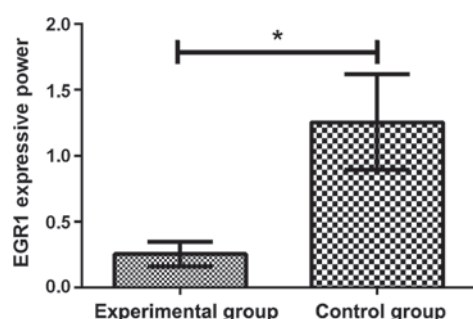


Figure 2. Expression of EGR1 in both groups of patients. EGR1, early growth response. * $P<0.05$.

Table III. Pearson's correlation analysis of the expression and clinical indicators of CD69 and EGR1.

CD69	r value	P-value	EGR1	r value	P-value
Lp(a)	0.352	0.01	Lp(a)	-0.394	0.01
hs-CRP	0.402	0.01	hs-CRP	-0.524	0.01

EGR1, early growth response; Lp(a), lipoprotein a; hs-CRP, high sensitive C-reactive protein.

of CD69 was, the higher the sensitivity and specificity were in evaluating the progression of the patient conditions. The AUC of the expression level of EGR1 in plasma was 0.933 (95% CI: 0.867-0.978), and the sensitivity was 89.7%, and the specificity was 79.2%. The lower the expression level of EGR1 was, the higher the sensitivity and specificity were in evaluating the progression of the condition of the patients. Finally, by the combined detection of CD69 and EGR1, it was found that AUC was 0.954 (95% CI: 0.887-0.982), and sensitivity was 90.5%, and specificity was 86.3% (Fig. 3).

Discussion

As the most common cardiovascular system disease, CHD is mainly caused by stenosis and obstruction of blood vessel lumen as a result of coronary AS. It is one of the major diseases of human health in the world, and statistics show that the incidence of CHD is increasing year by year (13). As the most common clinical cardiovascular disease in the

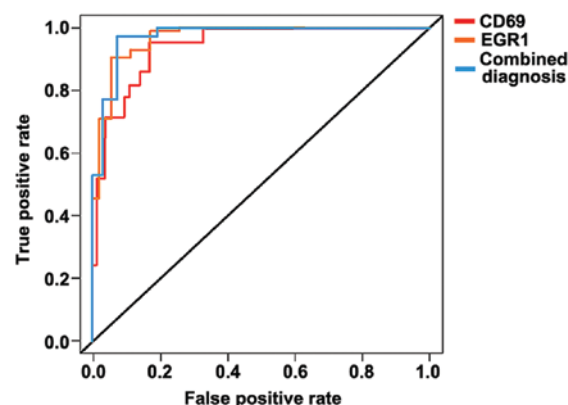


Figure 3. The ROC of CD69 and EGR1. EGR1, early growth response.

elderly, the untimely treatment of CHD will lead to disability and death, and it is also a disease with high mortality in the elderly (14). According to statistics (15), the number of patients suffering from cardiovascular diseases in China is as high as 290 million, accounting for >30% of the proportion of death, and the mortality and morbidity rate are increasing year by year. At present, the main diagnostic methods are cardiac stress test and coronary angiography. Cardiac stress test requires higher physical condition of patients, and coronary angiography is one of the most accurate diagnostic methods, but its high price increases the economic burden of many patients (16). Therefore, we need to find new diagnostic indicators to better diagnose the patient's disease and alleviate the patient's suffering by timely treatment to improve the patient's quality of life. In recent years, gene diagnosis of various diseases has become a popular method, and we found that there were differences in the expression level of CD69 and EGR1 in patients with CHD by screening from ceo database.

As a member of type II C plant hemagglutinin-like receptor family, CD69 can be activated by early leukocyte receptor induction, which is less expressed in the resting lymphocytes. After the activation of cells, its expression is induced rapidly, which can be detected on the surface of different activated leukocyte subsets (17). In addition to the immune response, the receptor has many other effects, such as synthesis and differentiation of cells, and regulation of inflammatory response (18). In the present study, through the detection of CD69 in peripheral blood of patients with CHD by flow cytometry, it was found that the expression level of lymphocyte subsets CD69 in CD3⁺CD4⁺T in peripheral blood of patients with CHD in experimental group was significantly higher than that in control group, which supported the result of gene chip screening. Moreover, in the study of Lei *et al* (19), it was found that the expression of CD69 could be an independent prognostic factor in patients with type 2 diabetes mellitus and CHD, which also supported the results of this experiment.

EGR1 has been named for existing widely in human cells and its ability to express rapidly. EGR1 belongs to one of the members of the immediate early family, and >30 members of the family all have zinc finger structure coding region and have high homology (20). There is a study showing that EGR1 has a complex signal pathway, which plays an important role in cell growth, differentiation, proliferation and inflammatory

response (21). As a pathological reaction caused by vascular stenosis and hemodynamic obstruction, CHD is composed of many factors such as inflammation, secondary thrombus and plaque rupture (22). In this study, by detecting the expression of EGR1 in plasma of patients, it was found that the relative expression level of EGR1 in plasma of patients in the experimental group decreased significantly, compared with the control group. Besides, in the study of Toutouzas *et al* (23), it was found that through RT-qPCR detection, the expression of EGR1 of patients with CHD in the blood stasis group and the non-blood stasis group was significantly lower than that in normal group, which also showed that there was a difference in the expression of EGR1 in patients with CHD. We speculated that the decrease of the expression of EGR1 might be due to the change of the patient's condition, which inhibited the binding of zinc finger structure coding region of EGR1 to downstream related genes, leading to the difference of expression of many biochemical factors, so as to result in the occurrence of CHD in patients. As a cholesterol macromolecule lipoprotein, [Lp(a)] plays an important role in the occurrence of CHD and it has been shown to be an independent risk factor for CHD (24). hs-CRP is a new independent prognostic indicator for the diagnosis of CHD in recent years, and its expression level is positively correlated with the pathological changes of CHD (25). In the present study, through Pearson's correlation analysis, it was found that CD69 and EGR1 were correlated with the expression of [Lp(a)] and hs-CRP of patients, which suggested that the expression level of CD69 and EGR1 was related to the severity of patients with CHD.

At the end of the study, the results of ROC curve analysis showed that the AUC of the expression level of CD69 in peripheral blood in the course of the disease of patients was 0.889 (95% CI: 0.822-0.958), sensitivity was 73.4%, and specificity was 86.5%. AUC of the expression level of EGR1 in plasma in the course of the disease of patients was 0.933 (95% CI: 0.867-0.978), sensitivity was 89.7% and the specificity was 79.2%, which also showed that the expression level of the two indicators was expected to be a new indicator for evaluating the progression of the conditions of patients with CHD.

However, the number of patients is small and the patients are all local. Whether there are regional differences in the results needs to be verified by a large number of samples. Therefore, we hope to increase our sample size and regional samples in future studies to prove and perfect the correctness and objectivity of this study.

In conclusion, the expression level of EGR1 in plasma of patients with CHD decreased, while the expression level of CD69 in peripheral blood increased, and both of them were related to the severity of the disease of patients, which could be used as an indicator to evaluate the progression of the condition of the patients.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JP designed the study and wrote the manuscript. JP and YX were responsible for sample collection and PCR. Both authors read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of People's Hospital of Hunan Province (Changsha, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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