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PRELIMINARY COMMUNICATION

DNA microarray study of genes differentiating acute myocardial infarction patients from healthy persons

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Abstract
Objective: Using oligonucleotide microarrays HG-U133A, we here studied the expression levels of genes that could differentiate between patients with myocardial infarction (MI) from healthy subjects, as well as to select among such genes those that seem crucial for manifestation of cardiovascular diseases.

Materials and methods: The microarray study was conducted using material derived from blood samples collected in 17 individuals.

Results: Analysis of gene expression data from 17 microarrays allowed identification of 28 genes strongly differentiating the examined groups.

Conclusion: The differentiating genes that we tracked down indicate possible linkage with atherosclerotis and could be a prognostic marker for development of cardiovascular diseases.

Keywords: Atherosclerosis, cardiovascular diseases, oligonucleotide microarrays

Introduction

Mortality due to cardiovascular incidents, including myocardial infarction (MI), is the leading cause of death in the developed countries around the world. It is no wonder that great research effort has been concentrating on a quest for finding mechanisms that lead to the development of these disorders, as well as possibilities of treatment and prevention at the molecular/genetic level.

Attempts have been made to identify genetic predispositions that lead to the development of atherosclerosis. This includes predispositions ascribed to genes encoding lipid metabolism-regulating factors (Incalcaterra et al. 2004; Licastro et al. 2004; Tobin et al. 2004), proinflammatory factors (Ameziane et al. 2003; Nojiri et al. 2003; Ye, Gale and Martyn 2003; Iacoviello et al. 2005), endothelium- and vascular wall-related factors (Muckian et al. 2002; Fatini et al. 2004), as well as blood clotting factors (Panahloo et al. 2003; Grove et al. 2004; Ott et al. 2004). Activation of inflammatory process in acute coronary syndrome (ACS) is connected with elevated activity of collagen-degrading enzymes (connective tissue metalloproteinases such as matrix metalloproteinase, *MMP*) as well as with decreased synthesis of their tissue inhibitors (such as tissue inhibitor of metalloproteinase, *TIMP*) (Libby 2001). Enzymatic degradation of elastin, which normally warrants integrity of arterial wall, can also favor instability of atheromatous plaque (von Bary et al. 2011).

With the advent of oligonucleotide microarray technology it became feasible to investigate expression level profiles of genes specific for various pathologies and to individualize patients' treatment depending on such profile.

We aimed to find, using oligonucleotide microarrays HG-U133A (Affymetrix), genes that differentiate patients with MI from healthy individuals, as well as to select from among such genes those that appear to be the most crucial for development of cardiovascular diseases.

Materials and methods

Materials

The proposal for this study was prepared according to the Declaration of Helsinki-based GCP guidelines. The proposal was endorsed by the Bioethics Commission

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of the Medical University of Silesia in Katowice on December 21, 2004 (see resolution NN-6501-223/I/04).

The study was based on the use of oligonucleotide microarrays. The hybridized cRNA was ultimately derived from total RNA obtained using mononuclear cells from peripheral blood of subjects involved in the study. The 17 individuals examined comprised 13 patients admitted to the Clinic of Cardiology with acute MI and, as controls, four healthy individuals. All subjects participating in the study were Silesian residents.

The patients enrolled in the study (on average 62.2 years old; nine male subjects and four female) had not been treated previously in relation to coronary disease. Acute MI localized to the anterior wall was confirmed by ECG, cardiac ECHO, coronarography and serum concentration levels of troponin, creatine kinase and its cardiac isoenzyme.

The control group included healthy individuals, two male and two females (previously untreated), who reported to the emergency department with chest pain. They were included in the control group based on interview, physical examination and results of laboratory tests (blood morphology/blood smear, erythrocyte sedimentation rate, lipidogram, serum glucose concentration, creatinine, electrolytes), and no changes in coronary vessel angiography or 64-row multidetector computed tomography picture, the latter assessing coronary artery calcification (calcium score). The experimental group was set up based on specific inclusion and exclusion criteria.

The following study inclusion criteria were established: age above 18, conscious consent, first-time cardiac incident, i.e. lack of previous treatment for coronary disease, typical coronary chest pain lasting less than 12 hours, ECG changes in ST-T segment and T wave, or block of left atrioventricular bundle branch. The exclusion criteria included: no consent to participate, acute or chronic inflammatory disease present at the time of admission and within 3 months preceding inclusion in the study, autoimmune disease, renal insufficiency, and diabetes.

Methods

The study was performed using oligonucleotide microarrays HG-U133A (Affymetrix). For molecular studies, peripheral blood mononuclear cells (PBMC) were used from which RNA was isolated according to a modified Chomczyński and Sacchi method, followed by RNA purification with RNeasy Total RNA Mini Kit, (Qiagen, Hilden, Germany). The obtained RNA served as a template for double-stranded cDNA synthesis, which, in turn, served to prepare biotinylated cRNA. The labeled cRNA was then fragmented and hybridized to HG-U133A gene chip. The microarray-hybridized cRNA was next labeled with streptavidin–phycoerithrin complex. Fluorescence intensity was analyzed using the GeneArray Scanner G2500A (Agilent, USA). The obtained data were normalized with RMA Express software.

The genes that differentiate MI patients from healthy controls were typed using the Bland–Altman method, using the following criteria: (i) differentiation of Signal Log Ratio (SLR) of particular transcripts in the transcriptome; (ii) minimum value of fluorescence level assuring usefulness of the chosen differentiating transcript in quantitative real time polymerase chain reaction (QRT-PCR) analysis; and (iii) the so-called "biological criterion" meaning a desired minimum value of SLR coefficient that a differentiating transcript must have (in our case $SLR_{min} = \pm 1$). Both absolute values of measured differences and SD values were considered. Good reproducibility of the method was obtained (<95% of the differences were within difference average ±2 SD).

Results

Microarray analysis

Total RNA samples isolated from mononuclear cell fraction of studied individuals' peripheral blood were converted into double stranded cDNAs and then into labeled cRNAs. The latter were subsequently examined using oligonucleotide microarray approach.

In order to obtain correct results of the performed experiments we compared averages and standard deviations of normalized fluorescence measurements of all transcripts present on the HG-U133A gene chips. In order to eliminate unwanted variability associated with the microarrays used, the data were normalized so as to obtain identical fluorescence intensity distribution for all the samples examined. Normalization was performed using RMA Express software.

The analysis did not reveal differences of background values in separate experiments; all microarray data obtained were thus included in the subsequent analysis.

First, we performed data clustering analysis. It was assumed that Euclidean distance is a measure of similarity describing differences between expression values in the set of the analyzed data. Clustering analysis was performed with the help of Cluster 3.0 software and the results were visualized with Java Treeview.

Cluster analysis

Using hierarchical clusterization we analyzed simultaneously the expression of 22,283 genes. Clustering analysis demonstrates a very homogenous separation of the examined dataset into patients with acute MI and healthy controls (C) (Figure 1).

No intragroup differences were seen, especially in the group of patients with MI status.

Bland–Altman analysis

Using Bland–Altmann method we singled out, from among 22,283 genes present on a HG-U133A microarray, the ones that differentiate between MI patients and healthy controls (Figure 2).

Discussion

Cardiovascular diseases, as mentioned earlier, are a major cause of hospitalization and death in the

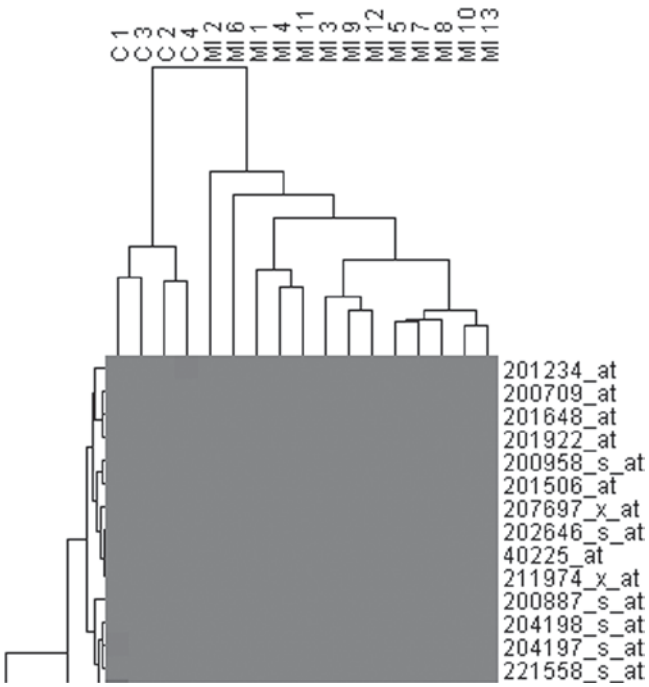

Figure 1. Clustering analysis of gene transcripts present on the HG – U133A gene chip. C1…C4 (controls); MI1…MI13 (patients with acute myocardial infarction).

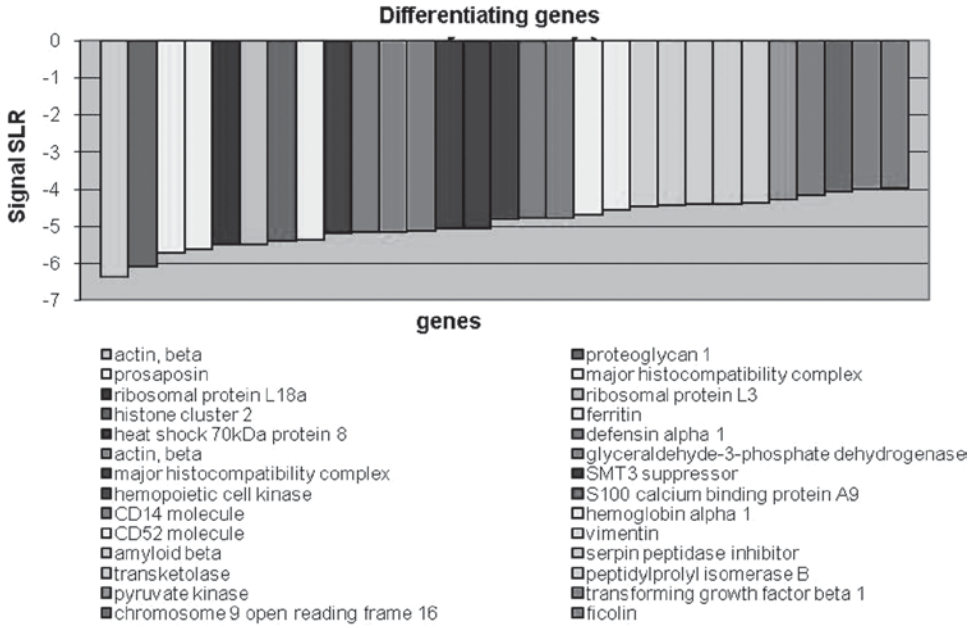

Figure 2. Genes differentiating healthy controls from acute myocardial infarction patients; selection from among 22283 transcripts present on HG-U133A oligonucleotide microarrays performed using Bland–Altman method.

developed nations. Knowledge concerning the roots of ACS, the consequence of atherosclerotic processes affecting coronary vessels, has substantially evolved in recent years. Dysfunctions of endothelium, state of inflammation, as well as intensified neurohormonal-type reactions involving endothelial cells' surface are believed to be the origin of such processes.

It has been believed for a long time that the major role in the rise of gradual changes in the vessels' architecture, characteristic for atherosclerosis, is played by cytokines. In the seventies and the eighties of the past century particular attention was paid to growth factors, especially those affecting muscular coat cells, the proliferation of which was believed to be the major cause of atherosclerosis (Ross 1981; Weissberg et al. 1996). In the nineties the focus of interest switched to immunomodulatory cytokine networks favoring rise of inflammatory state in blood vessels (Hansson et al. 2002). At present, it is known that development of atherosclerotic lesions may start in the first year of life, and even before birth (Weissber & Rudd 2002).

It is no wonder then that an ever increasing role in atherosclerosis is being ascribed to genetic factors. For many years, research effort has been concentrating on the quest for a gene that would be an ideal marker of acute coronary condition. It was demonstrated that the occurrence of certain variants of genes encoding several cytokines or factors crucial for atherogenesis does correlate with the frequency and intensity of the disease process. Morgan et al. (Morgan et al. 2007) attempted to show a statistically significant link between genotype and cardiovascular morbidity through searching medical databases and papers that appeared prior to 2005. These researchers investigated 85 polymorphic genetic variants and concluded that genes such as *ACE*, *AGT*, *AGTR1*, *ITGB3*, *F2*, *F5*, or *MTHRF* can be the markers of increased risk of ACSs. In turn, the study of Meier et al. (Meier et al. 2009), concerning collateral circulation index in ACS and non-ACS patients, revealed 76 genes that differentiated the examined groups. Among them are integrin family genes, growth factors and genes participating in angiogenesis.

Herein, we show the genes that differentiated acute MI patients from healthy individuals in our study; we point to genes linked with apoptosis (proteoglycan 1, ferritin), inflammation (S100 calcium binding protein A9, *CD14*, transforming growth factor β 1), immune response (defensin) and lipid transport (prosaposin) as taking part in the atherosclerotic processes. The results reported herein are preliminary; whether the reported gene expression changes indeed relate to post-MI remodeling, to macrophage activation or whether they are crucial for manifestation of cardiovascular diseases would require careful QRT-PCR scrutiny of a larger patient cohort.

Even though pathology of atherosclerosis has been studied in-depth for many years already, it should be obvious that search for unknown mechanisms of atherogenesis needs to be continued, together with deeper investigation of the already-known ones.

Conclusion

The selected genes that differentiate between MI patients from healthy individuals indicate relationship with atherosclerosis and could be a prognostic marker for development of cardiovascular diseases.

Acknowledgments

Molecular work was performed in Department of Molecular Biology, School of Pharmacy and Division of Laboratory Medicine, Medical University of Silesia in Katowice.

Declaration of interest

The research project entitled "Oligonucleotide microarray study of proinflammatory genes expression profile in mononuclear peripheral blood cells and right atrium cells obtained from atherosclerotic and ACS (unstable coronary disease, myocardial infarction) patients" and which was designed according to Declaration of Helsinki-based GCP guidelines was positively endorsed by the Bioethics Commision of the Medical University of Silesia in Katowice on December 21, 2004 (resolution NN-6501-223/I/04).

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