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Minireview

"TransEpiGen-omics" in cardiovascular disease research: Unraveling the genetic basis of complex diseases

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Summary. The genome, methylome, and transcriptome are functional components of a comprehensive network working in the background in support of our health. Thus, an integrative approach in combining and analyzing data from different sources and of different types is necessary in order to improve the understanding of biological processes and biological systems as a whole. In the past two decades, most candidate gene association studies in cardiovascular disease (CVD) have identified genes and variants that affect lipid levels, inflammation and the biology of the vascular wall. Further, a non-candidate-driven approach has yielded the definition of both biologically explainable and novel genes without a known biological background. In summary, the genetic susceptibility to CVD is mainly described by the influence of many common single nucleotide polymorphisms (SNPs), with the small effect size supporting the common disease/common variant hypothesis. Consequently, many integrative concepts were applied in order to distinguish functionally relevant genetic variants, especially noncoding ones. Expression quantitative trait loci (eQTL) analysis refers to the widespread regulation of gene expression mostly by *cis*-acting SNPs. MicroRNAs have also become interesting targets in both research and therapy in atherosclerosis and CVD. A number of miRNAs has been shown to have a role as risk factors for atherosclerosis progression, while some share their atheroprotective effect. The complex and multidimensional nature of cardiovascular risk factors and outcomes could additionally be resolved by research into epigenetic regulation. Distinct epigenomic patterns exist in key DNA elements (promoter CpG islands, intragenic CpG islands, gene bodies and H3K36me3-enriched regions) of the cardiac genome. Moreover, differential expression of each corresponding gene correlates with differential DNA methylation in heart failure. It is clear that the aim of cardiovascular -omics in the next decade is to find better algorithms to integrate as much as possible different types of data into the biological networks underlying the disease phenotype.

Keywords: cardiovascular disease, complex trait, gene expression, methylation, microRNA, single nucleotide polymorphism.

Introduction

Improvement of both the understanding and treatment of complex diseases is the main challenge of biomedicine and genetics in the 21st century. From the genetic point of view, the traditional genetic mapping methodology (e.g. linkage analysis, family studies) was not quite successful in resolving the genetic basis of complex diseases. Single nucleotide polymorphisms (SNPs) are accepted as the most common and stable form of genetic variation and have become valuable genetic markers in association studies of disease susceptibility, progression and response to therapy.

Complex trait variability is still a very intriguing field and recently it was shown that aside from classical estimations through twin and family studies, SNP-based heritability can be estimated from unrelated individuals (Yang et al. 2011). The methods for estimation of trait variation explained by genetic factors are constantly improving. An estimate of heritability from genome wide association study (GWAS) data is an emerging field and several biostatistical models have been used. They are designed to evaluate trait variability from any set of SNPs. It was recently found that common SNPs can explain a great amount of heritability previously assessed by a familial approach, for many com-

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plex traits in humans (Shi et al. 2016). Common SNPs are also confirmed to be useful and informative in case-control traits. It was also suggested that hidden causal variants could be missed due to imperfect tagging or allelic heterogeneity, i.e. multiple causality. Still, certain limitations of models currently applied to trait heritability estimation need to be overcome, e.g. the limited information of the linkage disequilibrium (LD) over the regions. Nevertheless, the use of SNPs from arrays shows bioinformatical potential in assessing complex traits and disease research.

Most of the association studies in cardiovascular disease (CVD) have identified the genes and variants affecting lipid levels, inflammation, vascular biology and thrombosis, but for the majority of them the results are not conclusive. On the example of studies performed on the Serbian population, we will summarize the most investigated genes affecting these processes: apolipoprotein E (Zurnić et al. 2014), apolipoprotein (a) (Dincić et al. 2005), lipoprotein lipase (Glisić et al. 2003); the renin angiotensin system gene – angiotensin-I converting enzyme (Stanković et al. 2002; Kolaković et al. 2012), angiotensin II receptors 1 and 2 (Stanković et al. 2003, 2016; Kolaković et al. 2016); matrix metalloproteinase-1, -3 and -8 (Djurić et al. 2008, 2011, 2012). Haplotype investigation brings to a higher level our understanding of cardiovascular genetics. Nevertheless, the integrative approach knocked on our door more than a decade ago. Integration of biological data includes methods of combining, manipulating and analyzing different types of data from different sources in order to improve the understanding of processes and biological systems as a whole. The more data obtained, the more new approaches and algorithms are necessary.

Genome wide association approach in CVD

It is now quite clear that GWA studies have led to the identification of many novel genes and variants in all pathologies, including cardiovascular, during the past ten years. The hypothesis of a common disease-common variant was thoroughly investigated in a non-candidate-driven approach and yielded the definition of both biologically explainable genes and novel genes without an established biological background. The great GWAS breakthrough in the field of cardiovascular genetics occurred in 2007 when several studies under the umbrella of the Framingham Heart Study were published, as well as the WTCCC study where only one novel CAD-associated locus was defined (WTCCC 2007). This was the significant signal present on chromosome 9p21.3, the strongest at rs1333049, although associations were detected for SNPs across >100 kilobases. This is the genetic region that is most robustly associated with cardiovascular disease, and most importantly, independently of traditional risk factors (Schunkert et al. 2008). It was associated with coronary heart disease (McPherson et al. 2007), myocardial infarction (Helgadottir et al. 2007) and atherosclerosis (Ye et al. 2008; Zivotic et al. 2016), presenting with the several SNPs that are in strong LD (rs1333049, rs10757278). CDKN2A (p16INK4a) and CDKN2B (p15INK4b) are genes closest to SNPs, and are mostly located in the long noncoding RNA, CDKN2AB (ANRIL). This association and the story after its discovery is a perfect example of a GWAS discovery followed by functional studies aimed at determining the biological explanation of this association. First, the regulatory potential of the region was impressive. It was discovered that the 9p21 region harbors 33 enhancers and represents a gene desert for predicted enhancers, which is six times denser than in the entire genome (Harismendy et al. 2011). It was discovered that the expression of all three of the abovementioned genes is positively correlated, except for certain ANRIL exons, with CDKN2B. After knockdown of ANRIL, the expression of CDKN2B was significantly upregulated, as well as the proliferation of vascular smooth muscle cells (Congrains et al. 2012). The SNPs in the region independently affected the expression of all genes, with particular emphasis on fact that disease-associated SNPs affect ANRIL expression, which suggests that ANRIL modification might have the greatest impact on disease susceptibility. Other mechanisms suggested to be responsible for ANRIL 9p21 SNPs association include miRNA and epigenetics, and a complex transcript isoform structure (Folkersen et al. 2009; Liu et al. 2009; Murray et al. 2016).

Towards calculating a genetic risk score

The trend that came after the GWAS was the search for the "missing heritability", since only a limited percentage of it was explained by GWAS. Accordingly, the rare variants that might have the larger effect become the focus of further research (rare variant-common disease hypothesis). Actually, according to today's knowledge, 1% of the human genome is transcribed into mRNA and translated into proteins. The template for noncoding RNAs and regulatory regions involved in gene expression makes up an additional 5%. The rest was referred to as "the dark matter of the genome" (Blaxter 2010). According to Venter's report from 2007, 4.1 million sequence variants, which include ~3.5 million SNPs, affect ~44% of annotated genes (Levy et al. 2007). Each genome has a 50-100 variants that are linked to inherited disorders and ~300 variants defined as "loss of function". Calculated using SNPs it points to a great difference between genomes.

By putting this knowledge together with a few examples of rare allele associations with a complex clinical phenotype such as CVD, we can only search for the additive effects and interactions of multiple variants (e.g. alleles) with varying size in their effects.

The GWAS in coronary artery disease (CAD) up to 2013 reported the presence of 31 loci associated with CAD risk, which explain about 10% of its heritability (The CARDIoGRAMplusC4D Consortium 2013). New results indicate

that complex traits involve large numbers of causative alleles with a relatively small effect size (Yang et al. 2011).

Currently the largest consortium that investigates the genomics and genetics of CAD, the CARDIoGRAMplusC4D Consortium, has reported an additional 15 significant GWAS loci for CAD, plus 104 variants strongly associated with the phenotype (The CARDIoGRAMplusC4D Consortium 2013). They also created five interaction networks with 223 candidate genes involved in CAD. The pathways in most significant networks were linked to lipid metabolism and inflammation, which emphasizes the role of these processes in CAD. It was a novel way to confirm key processes in atherosclerosis described 15 years ago (Hansson et al. 2002). Macrophages take up oxidized lipids and create foam cells that secrete proinflammatory cytokines and matrix metalloproteinase, creating the inflammatory loop and stimulating vascular smooth muscle cells (VSMC) to proliferate and migrate into the plaques. Blood pressure remains a trait of never-ending interest, and was also confirmed with 5 SNPs associated with it in the same study, but none of the variants was associated with diabetes. Nevertheless, the 150 SNPs with GWAS significance in the largest sample of Europeans and South Asians to date provide a basis for calculating the genetic risk score for CAD. Further, the capability of GWAS to examine the genetics of complex diseases has been improved by the 1000 Genomes Project. One hundred eighty-five thousand CAD cases and controls were included in the meta-analysis cross-examining the 6.7 million common variants and 2.7 million low frequency variants with minor allele frequency between 0.005 and 0.05. Ten novel CAD loci were identified in candidate genes after the establishment of new connections with processes in vessel walls (Nikpay et al. 2015). Although the loci were novel, their role in atherosclerosis highlighted processes such as cell adhesion and leukocyte and VSMC migration and senescence, which are well known, suggesting a crucial role of arterial wall biology in CAD pathogenesis. The conclusion of the study indicated that it is unlikely that low frequency variants and synthetic associations and insertion/deletions can explain a significant part of missing CAD heritability. This study has shown once more that the genetic susceptibility to CAD, as well as other complex diseases, is mainly characterized by the influence of many common SNPs, with the small effect size confirming the common disease/common variant hypothesis.

Gene regulation

It became clear that risk alleles cluster within or close to functionally annotated regions. The biology of gene regulation is currently a field of intensive research. Many complex processes underlie the basic processes of transcription and translation. Increased knowledge of these processes is the ultimate aim of research into the genetic basis of complex human diseases. The keywords in this area are: transcription factors, promoters, chromatin remodeling, chromatin modifications and microRNA. The cherry on top in the gene modification processes, without changing the DNA sequence, is epigenetic modifications. The expression of genes in a parent-of-origin-specific manner, genomic imprinting, affects a relatively small number of genes, but could significantly influence the development of disease and disease risk.

Functional annotation of SNPs though transcriptome analysis

The majority (88%) of SNPs shown to be associated with complex diseases by GWAS are noncoding variants (Hindorff et al. 2009). The biological explanation of such associations is more challenging than of those for disease-associated variants localized to the coding regions of a gene.

Many integrative concepts are applied in order to distinguish functionally relevant noncoding SNPs from their more numerous irrelevant counterparts. One of them is expression quantitative trait loci (eQTL) analysis, which investigates whether the expression of a gene is associated with proximal SNPs (Folkersen et al. 2010; Zhu et al. 2016). During the past few years, the strong enrichment of SNPs for eQTL was demonstrated, supporting the regulatory role of SNPs in complex diseases. The hypothesis that genetic variants in promoters and enhancers influence gene expression was confirmed by GWAS that have used gene expression as a quantitative trait and have indicated the widespread regulation of gene expression mostly by *cis*-acting SNPs. The limitation of eQTL analysis based on SNP GWAS is the absence of an evaluation of the structural genetic variations' (copy number variations, balanced rearrangements and mobile-element insertions) influence on gene expression. Recently, using whole genome sequencing it was shown that structural variations have causality in eQTL of 3.5-6.8%, which is more than expected. Results also suggested that structural variants mostly act through regulatory mechanisms since the greatest enrichment was detected in the regions of enhancers, up- or downstream of transcripts, and regions rich in transcription factor binding elements (Chiang et al. 2017). The tagging relationships of SNPs and structural variants via LD is an important consideration and is now resolved as results suggest different percentages of tagging between them (from 58%-79%) for gene expression as a quantitative trait.

Different models of the causality of genetic variants in the association between gene expression and phenotype were proposed. The cell type specificity of eQTL is another relevant factor in such approaches. The tissue that is considered relevant for certain phenotypes or diseases is not always known and can be misunderstood, as for example adipose tissue in the body mass index. New results show that the expression in the brain is more relevant for this trait than in adipose tissue (Locke et al. 2015). Nevertheless, the major-

ity of eQTL studies use cell culture. A few years ago, the 166 SNPs associated with traits that carry the risk for cardiovascular disease were investigated in association with gene expression levels in different tissues: liver, aorta and carotid plaque (Folkersen et al. 2010).

The difference in the strength of association was found on the basis of distance between SNPs and the related genes. Among all analyzed SNPs, 47 were associated with the expression level of a nearby gene. One of the important conclusions was that genes located next to risk-SNPs do not by default participate in the disease mechanism. In addition, even SNPs that were not in closer proximity to the associated gene (and there were no SNPs in LD > 0.4, which is rather moderate) could influence gene expression (Folkersen et al. 2010).

A further direction of research is to link, if possible, the genetic variants to the protein level, either in the plasma or a specific tissue. Such a study was recently performed using large-scale measurements of hundreds of plasma samples in combination with genotyping in order to detect SNPs that influence cardiovascular plasma biomarkers (Folkersen et al. 2017). The examined SNPs explained 10% or more of plasma protein variability for 23 proteins (Folkersen et al. 2017). The *cis*-acting SNPs are likely to be the first step in the regulation of not only gene transcription but also protein synthesis.

MiRs in cardiovascular pathology

The complexity of posttranscriptional regulation by microRNAs (miRs) is far from being fully understood. MiRBase v21 (Kozomara and Griffiths-Jones 2014) suggested the existence of more than 2500 miRs. The ability of a single miR to interact with many different genes and to share the same target genes with other miRs makes the estimation of their role in complex human diseases even more complicated. We recently reported that a common SNP in the angiotensin II receptor type 1 (rs5186) significantly positively affected both AT1R mRNA and protein expression, as well as miR-155 in human carotid atherosclerotic plaques (Stankovic et al. 2016). MiR-155 is a good example of a well-studied miR, but its role in atherosclerosis has yet to be elucidated. A pro-atherogenic potential of this miR was shown by enhancement of vascular inflammation by macrophages in apoE$^{-/-}$ mice (Nazari-Jahantigh et al. 2012) as well as atheroprotective regulation of immune cells of LDLR$^{-/-}$ mice fed a high fat diet (Donners et al. 2012). The miRs are interesting targets for both research and therapy in atherosclerosis and cardiovascular disease. A number of miRs have been shown to have a role as risk factors for atherosclerosis progression, while others share their atheroprotective effect. We recently applied a bioinformatical prediction approach in order to define potential miRs with both functions. After extensive data mining of miRs in atherosclerosis we found that our methodology was successful in both recognizing previously described miRs in atherosclerosis, as well as in identifying new ones (Jovanović et al. 2014). Post-myocardial infarction (MI) ischemia and cardiac remodeling triggers many processes inside the heart, including inflammation and transcriptome changes. MiRs were recognized as useful markers of consequent changes, but also as early markers of the event itself. Phenotyping the changes in post-MI cardiac cells, cardiomyocytes, fibroblasts and endothelial cells revealed that they are partially driven by miRs (Fiedler 2013). The mechanisms by which miRs are transferred into the circulation and urine allows the assessment of their abundance in distant tissues (Cheng et al. 2012). Although many companies are developing miR-based therapeutics, the priority is the validation of their role in cardiovascular pathologies.

Cardiovascular epigenomics

Aside from a candidate gene approach focused on single gene methylation, epigenome-wide association studies (EWAS) allow a systematic assessment of many CpG sites across the genome in relation to the phenotype of interest (Rakyan et al. 2011). Many genome-wide human methylation datasets are now publicly available for numerous tissues along with basic phenotypic information. The complex and multidimensional nature of cardiovascular risk factors and outcomes could be additionally resolved by research into epigenetic regulation (e.g. histone modification, posttranscriptional silencing by miRs, long and short noncoding RNA and mitochondrial DNA methylation), and translated to a target for intervention in cardiovascular medicine. In blood samples from individuals with a history of MI, CpG methylation patterns are altered at many genomic loci that have been previously linked to CVD (Rask-Andersen et al. 2016). An EWAS that aimed to identify CpG sites at which DNA methylation levels are associated with increased blood lipid levels revealed the association of HDL-C levels with the methylation of a CpG site near DHCR24, a protein-coding gene involved in cholesterol biosynthesis (Braun et al. 2017). Furthermore, EWAS and GWAS integration can identify novel genotype-epigenotype interactions within disease-associated loci (Rakyan et al. 2011).

Large epigenetic consortiums have been formed (Braid et al. 2017) to obtain different methylation data. It is very important to obtain data at birth and to compare them prospectively with data obtained later in life in order to determine epigenetic contributions to health and disease (Rakyan et al. 2011; Braid et al. 2017).

As life style and environmental factors might significantly influence methylation, even *in utero,* such data are invaluable. For example, *in utero* exposure to maternal cigarette smoking and maternal folate intake led to DNA methylation changes shown to be related to CVD outcomes later in life and even congenital heart disease (Heijmans et al. 2008; Božović et al. 2015; Lee et al. 2015; Murray et al. 2016). A large body of epidemiological studies has linked lifestyle and

environmental factors to DNA methylation and pathways dysregulated in CVD, such as blood pressure, heart rate variability, blood coagulation, etc. (Bellavia et al. 2013; Tarantini et al. 2013). In recent years, research on cardiovascular epigenetics has been based on different biological models in animal and epidemiological studies. In apoE-null mice, DNA methylation changes were observed in both peripheral blood leukocytes and the aorta prior to the formation of vascular lesions (Lund et al. 2004). When human failing hearts were compared to normal heart tissue, differential methylation, as well as differential expression, was observed (Movassagh et al. 2011; Olsen et al. 2017).

Different methodological approaches have also been applied. Methylation of the long interspersed nucleotide element-1 (LINE-1) was shown to be a good indicator of the global DNA methylation level. The hypomethylation of satellite repeat elements correlated with up to 27-fold upregulation of the corresponding transcripts in end-stage cardiomyopathic hearts (Haider et al. 2012). Distinct epigenomic patterns exist in important DNA elements (promoter CpG islands, intragenic CpG islands, gene bodies, and H3K36me3-enriched regions of the genome) of the cardiac genome: in human end-stage cardiomyopathy, mostly in genes related to myocyte apoptosis, fibrosis and altered contractility (Movassagh et al. 2011), as well as in MI, mostly in myofibroblast differentiation pathways (Olsen el al. 2017). Moreover, the differential expression of each corresponding gene was found to be correlated with differential DNA methylation in the blood of heart-failure (HF) patients (Li et al. 2017). Besides tissue-specific DNA methylation, novel findings point to the importance of the cell-type specificity of DNA methylation, further deepening the complexity. The adjustment for cell-type mixture in epigenetic association studies should be provided (Houseman et al. 2012).

Future studies are needed to investigate more thoroughly whether observed DNA methylation changes in fact translate to downstream biological changes (e.g. changes in chromatin accessibility, mRNA and protein expression).

Conclusion

The aim of cardiovascular -omics in the next decade is to create even more -omics data and to integrate them into the biological networks that underlie the disease phenotype. Translation into clinics and opening new therapeutics avenues is the ultimate aim.

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