Cent. Eur. J. Med. • 4(1) • 2009 • 1-10
DOI: 10.2478/s11536-009-0012-y

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Central European Journal of **Medicine**

Microarray technology in the study of genetic determinants of cardiovascular diseases

Review Article

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Received 11 December 2008; Accepted 14 January 2009

Abstract: Microarray, a miniaturized glass slide or membrane with immobilized DNA probes, is a powerful tool for the analysis of mutations, gene expression and sequencing. This technique requires chip (glass slide or membrane) fabrication, preparation of probes and labelled targets, hybridization and data analysis. Microarrays give the possibility to evaluate a wide spectrum of candidate genes, to simultaneously observe interaction of genes, to detect polymorphisms within genes and identify therapeutic targets. Coronary artery disease being a major cause of death, is a disorder influenced by either genetic or environmental factors. Microarray analysis of gene expression can be used to identify genes involved in disease progression and in disease reduction. Chips also allow for the throughput and simultaneous analysis of a great variety of cell types such as cardiomyocytes, monocytes, macrophages, smooth muscle, endothelial, and fibroblasts and chemical mediators involved in cardiovascular disease pathology, their interactions and cumulative effects.

Keywords: *Cardiovascular disease • Chip • Microarray • Single nucleotide polymorphism (SNP)*

1. Microarray – new design, old concept

Microarray, a miniaturized glass slide or membrane with immobilized DNA probes (Figure 1), is a powerful tool for the analysis of mutations, gene expression and sequencing [1] that allowed for significant progress in biological science. The method exploiting the preferential binding of complementary single-stranded sequences of nucleic acids [2] is well known and widely used for many years. The process of printing PCR amplicons onto a coated glass slide was originally described by Patrick Brown and co-workers [3-4] at Stanford University. In cooperation with Mark Schena and Ron Davis working for Affymetrix, he created the basics of traditional microarray technology [5]. The oligonucleotide microarray was first developed by Stephen Fodor and his colleagues in the early 1990s [6-7] and nowadays chips based on this technique are commercially available from Affymetrix (Affymetrix Inc., Santa Clara, CA). DNA microarrays, which are basically a miniaturized but high-throughput form of dot blot [8], consist of five crucial components: the chip itself, a spotter or device for photolithography, a fluidic system allowing for chip hybridization, a scanner for reading the chip and a sophisticated computer programs to interpret the results [3]. This technique requires chip (glass slide or membrane) fabrication, preparation of probes and labelled targets, hybridization and data analysis [8].

2. The microchip

The microarray chip is a small membrane or glass slide with hundreds of thousands of microscopic spots attached in fixed positions. Each spot contains a huge number of copies of identical sequences. Microarrays are often manufactured on microscope slides owing to their low inherent fluorescence. Glass surfaces of slides have to be modified in an appropriate way to obtain a more hydrophobic surface that maximizes the adherence of applied biological samples and reduces

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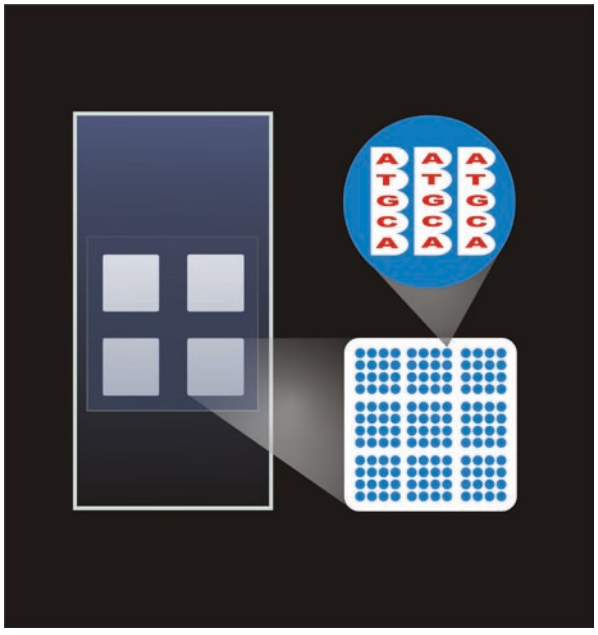
Figure 1. The chip scheme.



the spreading of spots [9]. Commercially available slides are coated with primary amine groups [SuperAmine, Telechem International Inc.] or primary aldehyde groups [SuperAldehyde, Telechem International Inc.] linked covalently with the glass slides, poly-L-lysine, aminosilane [10], streptavidin (used for capturing biotinylated molecules) [Xenopore], nickel NTA (used for capturing histidine labelled molecules) [Xenopore], agarose film [11] and nylon membranes (Cast TM Slides) [Schleicher&Schuell]. Being positively charged in neutral pH, amine and lysine groups link with the negatively charged phosphodiester backbone of native DNA by relatively weak ionic interactions. Additional and much stronger covalent linkage between thymidine rests of DNA and the amine groups on the surface is induced by the appropriate dose of UV radiation or heat [7,12]. Reactive aldehyde groups form covalent bonds with $-NH_2$ groups of DNA that are further stabilized by dehydratation causing the formation of Schiff's base-like molecules. Covalent binding allows for application of stringent wash conditions that permit diminishing the background and increasing the sensitivity of reaction [12]. Free reactive groups on the surface of the slide are blocked (usually by succinic anhydride) to avoid potential binding of target sample applied in the next step. The reduction of non-specific binding is obtained either by forming amide bonds between one of the carboxyl carbons and lysine for example or by creating negatively charged surfaces [13]. Accuracy and reproducibility of experiments with microarrays slides coated with amine, aldehyde or lysine groups may be of low quality due to the streaking of spots resulting in formation of comet tails, lack of homogeneity, and poor efficiency of hybridization [7,12]. DNA arrays in microbial systems can also utilize membranes (SigmaGenosys, Eurogentec), microelectronics (Nanogen) and polyacrymide gels instead of glass slides [8].

3. Probes for microarray

The kind of biological sample attached to the solid support depends on the type of microarray. There are three types of probes for microarrays:

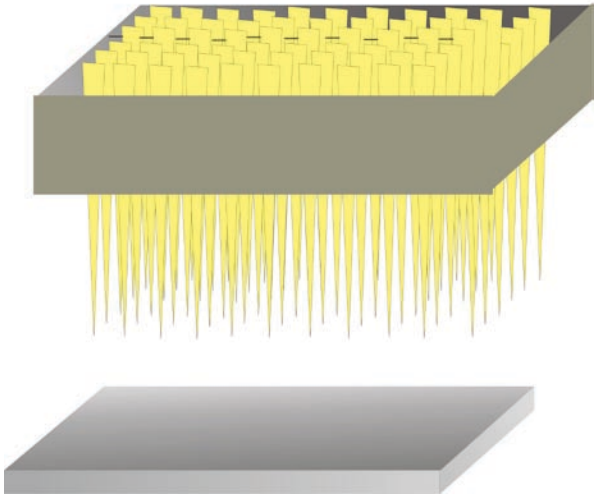
- DNA-based array – partial or complete cDNA or genomic DNA that should identify particular single genes or exons in the genome representing as many unique transcripts as possible in expression analysis and representing all genes suspected of being differently expressed in various states in comparative genomic hybridization,
- Oligonucleotide-based array – chemically synthesized oligonucleotide sequences (20-80 mers) representing many possible variations of various genes are used for SNP/polymorphisms analysis,
- Protein-based arrays – proteins, peptides and other biomolecules (antigens) attached to the chip surface are used for studying immune responses, drug actions, specificity and pathogenesis of autoantibody responses and identifying human autoantibodies [14].

3.1. DNA-based array

cDNA molecules of about 500-5000 bp length immobilized on a glass slide are obtained by reverse transcription of messenger RNA. An amplification step is usually required to obtain enough DNA probe for the array. The product of amplification should be purified from unincorporated nucleotides and primers for better efficiency of binding to the solid support [4].

Selection and deposition of probes, suspended in high concentrated salt solutions or in other denaturing buffer, are the crucial steps of microarray fabrication [12]. In the case of DNA-based microarrays, drops of solutions, each containing one type of DNA sample, are placed on microscope slide by an arraying robot. Spotting is achieved by mechanical sample deposition with the use of tin pins (physical contact of the pin with the slide) or ink jetting (a miniature nozzle with a piezoelectric oscillators and electrode guidance system expel the liquid) [5]. (Figure 2). Highly precise robots can produce quickly a regular grid of spots deposited at density depending on the arraying system (reaching

Figure 2. Sample deposition.



up to 10,000 spots/chip) [5] covering a surface small enough to hide under a standard slide coverslip [15]. The DNA spots are 100-200 μm or less in size [5].

3.2. Oligonucleotide-based array

Oligonucleotide chips are manufactured in two ways. Oligonucleotides are synthesized either in situ on a slide by means of photolithography or from short strands of pre-synthesized DNA deposited on a specially-prepared microscope slide. The idea of in situ synthesis technique is borrowed from the computer chip industry [5]. To prepare quartz wafer for in situ synthesis they are washed to guarantee uniform hydroxylation of the surface and then incubated with silane molecules followed by attaching the linker. Opaque mask covers the whole slide except for a number of 18-20 μm^2 windows through which the beam of light enters. Ultraviolet light removes protective groups from linker and nucleotides, activating parts of the chip not covered by a mask (Figure 3). Incubation with a solution containing one of four photosensitive hydroxyl-protected deoxynucleotides leads to its binding in a proper place [16]. These precursors are tethered at the 5' end and have a reactive 3-OH end after deprotection. Because some of new nucleotides may fail to attach to the activated molecules, a capping step is required [16]. Afterwards, another mask is placed over the slide to begin the next round of deprotection and coupling. Side chains of nucleotides are protected to prevent them from the formation of branched oligonucleotides [16]. The cycle is repeated with different masks, which results in obtaining a slide carrying up to 400,000 various groups of oligonucleotides (20-25 bases) with known sequences placed in highly determined positions [16-17]. NimbleGen modified the photolithography technique by introducing a system of very thin mirrors controlling the path of the light and determining the place of DNA synthesis. Replacement of masks allows for chip redesigning and lowers the chip costs [5]. When the slide is prepared, the hybridization step begins. Solution of labelled targets is pooled over the slide and complementary sequences bind to each other.

3.3. Protein-based microarray

Protein chips usually contain immobilized specific antibodies for high-throughput protein profiling. It is also possible to make arrays of full-length, functional proteins from a library of expression clones [18]. Such arrays allow for study of the interaction between proteins, measurement of ligand binding and immunoassays [11,19-20]. Protein arrays are commercially available from Ciphergen Biosystems Inc., Fremont California.

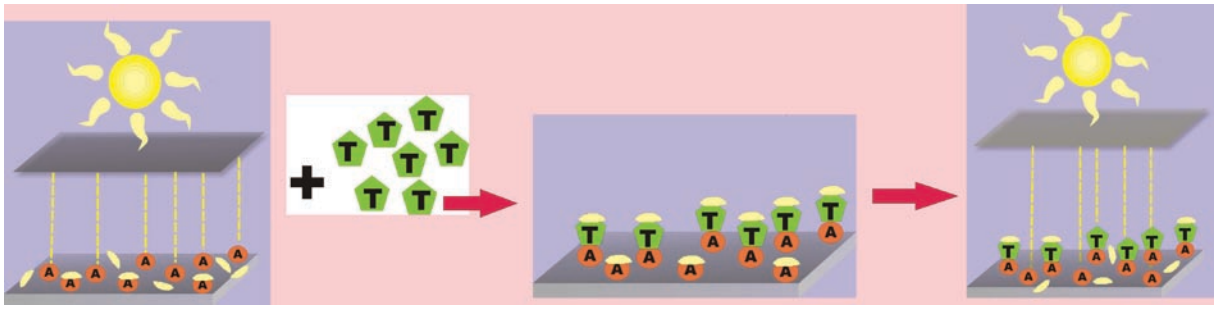
4. The basic types of microarray assays

There are three basic types of microarray that differ not only in the kind of immobilized sample but also in the type of controls and information derived from the chip [21].

4.1. Comparative genomic hybridization (CGH)

The first type is comparative genomic hybridization array assaying the relative representation of RNA species in

Figure 3. Photolitography procedure.



two samples. CGH is commonly used for comparing gene transcription in two or more different kinds of cells (i.e. diseased and healthy ones), for differentiating the genes expressed at different stages of cell cycle and for monitoring expression changes in response to various stimuli. It has wide applications in tumour classification and risk assessment, and identification of mutations in β-thalassemia patients [22] for example. In this type of array the mixture of target molecules contains fluorescently labelled cDNA or genomic DNA harvested from both normal (control) and diseased (sample) tissue. The type of genes analysed with this method depends on the choice of probe DNA. To prepare target cDNA, total mRNA is isolated and reversely transcribed into more stable cDNA. In the past, while producing cDNA, scientists usually took advantage of the fact that most mRNA has a poly-adenine tail and they used oligo(dT) primers so that the reaction started from the poly(A) tail. However, due to the fact that cDNA is not produced from all mRNAs with the same efficiency and that some mRNAs may be reversely transcribed only in parts too small to bind to the probe, such a method led to the reverse transcription bias that makes quantitative comparison between different mRNA samples on one array impossible [15]. To reduce this bias, application of random primers is recommended.

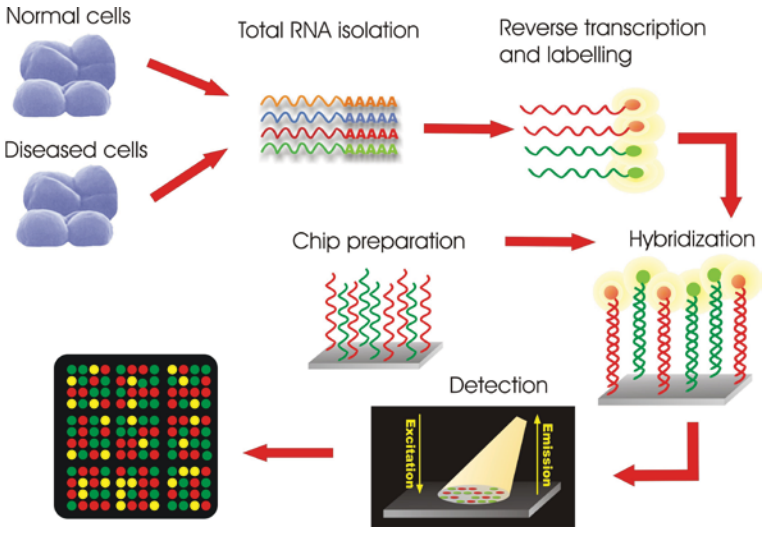
In comparative genomic hybridization, differently coloured dyes are used to label DNA so that different samples can be told apart. The amount of fluorescent dye molecules that labels each cDNA depends on its length and sequence composition, which means that fluorescent intensities coming from different cDNAs cannot be quantitatively compared [15]. In order to equalize the total concentrations of different cDNA targets, its solutions are diluted to have the same fluorescent intensity [15]. Such proceeding unjustly assumes that total amount of mRNA in each tested cell population is the same and that the amount of light emitted by each fluor is connected with its concentration [15]. Labelled targets from different samples are mixed and poured on the slide in order to determine the identity and the abundance of complementary sequences. In optimized conditions complementary sequences of targets and probes hybridize with high specificity according to Watson and Crick base pairing. Optimal hybridization conditions including pH, salt concentration, and temperature as well as probe selection are often calculated using computer models. In each spot there are enough probes for every complementary target to hybridize without any interference [15]. The longer the complementary fragments are, the stronger they attract each other [2]. In CGH and expression analysis, hybridization between a probe and a target allows for relative measurement of a particular sequence abundance in the target population. By comparing many hybridization patterns, the identification of differently expressed mRNAs is possible. If the expression of a gene in a particular state is increased, larger amounts of sample DNA, in comparison to control DNA, will hybridize to the same spots, resulting in greater fluorescence intensity. Monitoring the amount of fluorescence of the dye associated with each DNA position on an expression chip allows for the determination of the abundance of every represented mRNA species. In CGH and expression analysis hybridization, the relative representation of genes in samples is acquired by measuring the ratio of fluorescence intensities of labelled targets. The scanning device records light intensities coming not only from the probes bound to their complementary sequences in the spot, but also from the molecules mismatched or attached to the glass slide (background) [9]. Attempts to rule out variability in labelling and detection efficiency can be conducted by the application of controls, such as externally added sequences, reporter genes or total fluorescence for each sample [1]. Harvested information is stored for computer image analysis and statistical evaluations [9] (Figure 4).

4.2. Expression assays

The second type of DNA microarray is known as the expression chip. Expression microarrays are used in diagnosing and treating diseases linked to particular genetic expressions, such as some forms of cancer, drug and therapy development and monitoring drug response. Monitoring the expression levels of thousands of genes at a time provides scientists with insight into cellular processes and responses that cannot be obtained with the usage of former methods. Expression patterns can be used for studying various cell pathways, regulatory mechanisms and signalling [17].

In expression analysis, cDNA or cRNA targets [16] derived from experimental sample, hybridize on a chip with immobilized probes representing all possible genes. In order to detect target cDNA, it must be labelled with a reporter molecule. Fluorescent dyes such as rhodamine, fluorescein, Cye3-dUTP (540 nm, green light) and Cye5-dUTP (650 nm, red light) or radioactive agents can be used as reporters. Cye3-dUTP and Cye5-dUTP dyes are usually used together because they have different excitation and emission spectra. They are also photostabile and display high incorporation efficiencies [9]. Radioactive target is generated by the incorporation of [33P] or [32P]dCTP. Genomic DNA is commonly labelled by a nick translation, random priming with Klenov polymerase and direct chemical tagging [13]. Fluorescence labelling allows for the detection of <10μg

Figure 4. Comparative genomics hibridization scheme.



of total RNA while as little as 0,1µg RNA is enough for radioactive label detection [23]. The method applied for signal detection depends on the type of label used in an experiment: fluorescent dyes are scanned with the use of confocal laser scanners, radioactive labels with a phosphoimager or a simple radiography film [15], and enzymatic labels with a spectrophotometer [5].

4.3. Single Nucleotide Polymorphism assays

The determination of single nucleotide polymorphisms (SNP) playing a casual role in common DNA variation will help to assess an individual's risk of developing specific diseases. Oligonucleotide microarrays can track either length variation polymorphisms caused by a number of tandem repeats or single nucleotide polymorphisms brought about by insertions, deletions of single bases, transversions and transitions. Single nucleotide polymorphisms, in coding and regulatory regions, often change the individual's susceptibility to complex and multifactorial diseases [24] and also influence the response to drug treatment. Retrieving information from SNP chips with the usage of such techniques as genetic linkage mapping or association analysis will allow not only for the identification of SNPs that are the predictors of disease but also for the development of diagnostic screening tests and for early diagnosis of illness [5]. Personalized medicine aimed at tailoring appropriate treatment for each individual will probably be developed due to SNP analysis [5].

To determine whether or not SNPs are present in an individual's genome, his labelled DNA probe is hybridized to oligonucleotides on the chip. In typical Allele-Specific Oligonucleotide (ASO) hybridization technique, the differentiation between SNP alleles is made on the basis of differences in thermal stability between perfectly matched and mismatched allele-specific oligonucleotide probes and its DNA target sequence [25]. Sample DNA hybridizes with much greater frequency to specific SNP. The specificity of genotyping, obtained by differential hybridization with ASO probes, depends strongly on the nucleotide sequences flanking the SNPs as well as on the conditions of this reaction (temperature and ionic strength) and that is why multiple ASO microarray assays are performed with numerous "redundant" sets of probes [25]. The presence of a positive signal in a position representing a particular SNP confirms its occurrence in an individual's genome, and the lack of signal means that this SNP is absent [1].

To obtain more reliable results, many laboratories introduced some improvements of the basic microarray technique. Better distinction between SNPs alleles in comparison to ASO hybridization methods and limitation of useless probes can be obtained by the application of DNA-modifying enzymes (such as DNA polymerases and DNA ligases). Techniques based on high-sequence specificity of enzymes are more reliable than those using hybridization of complementary sequences [26]. While being independent of the DNA sequence composition, single-base primer extension and ligation methods allow for the genotyping of a great variety of SNPs in the same reaction conditions [25]. One of such techniques is efficient and high-throughput genotyping, based on the extension by reverse transcriptase of two allele-specific primers having 3'-nucleotide that determinates the allelic variant of SNPs [26-27]. D. O'Meara *et al.* [27] described an apyrase-mediated allele-specific extension method. This technique takes advantage of the fact that DNA polymerase acts much slower while extending a

mismatch primer instead of a perfectly matched primer. Certain mismatch configurations are known to yield extensions that hinder proper discrimination. Apyrase exploits this delay and degradates these nucleotides before extension [27]. In another technique, named Molecular Inversion Probe Assay, two probes hybridize to target DNA, leaving a single-base gap between them. This gap is filled with an appropriate nucleotide in a single base primer extension reaction. After the circularization of a probe by ligating its ends, it is amplified in PCR reaction. Amplicons are further cleaved, labelled with two or four fluorophores and analysed on a microarray [25]. Labelled RNA targets, obtained in reverse transcription of PCR products, are interpreted with array scanners and software. This single-step procedure ensuring robust genotyping not only allows for the usage of a single detection reaction with a single fluorophore but also for the application of less expensive pre-synthesized oligonucleotides [5]. Henk van Damme (PamGene, Boxtel, The Netherlands) presented a flow-through biochip technology (PAM microarray) which resolves the problem of the limitation of target diffusion by forcing them through a porous array. Apart from being fast (hybridization process can be completed in a few minutes), it is also very selective and sensitive (allows for the discrimination and the analysis of a point mutation) [28]. Technique based on the labeling of oligonucleotide targets with a “high-density” nanoparticle (Nanosphere Inc, Evanston, Illinois, USA) allows for 3-fold increase in the sensitivity of allele discrimination compared to the methods using fluorophore-labelled targets. The application of oligonucleotide-modified gold nanoparticle probes results in a significant alteration of the melting profiles of the targets from an array substrate, permitting the discrimination of an oligonucleotide sequence from targets with a single nucleotide [28].

5. Human samples

Human samples for arrays may be obtained by the amplification of clone inserts from human cDNA libraries or by synthesizing oligonucleotide sequences on the basis of the known expressed sequence tags (ESTs) [15]. Microarray probes are often chosen directly from such databases as GenBank, UniGene, and dbEST [9]. The reduction of complexity of the human genome before SNP genotyping can be obtained by the cleavage of genomic DNA with restriction enzymes, the ligation of obtained fragments with common adapters having no homology with the known genome sequences and amplification with universal primers complementary to adapter sequences [25,29]. This method of amplification of thousands of DNA fragments with one pair of primers allows for 50 times the reduction of human genome complexity while using an XbaI enzyme [25]. Introduction of a second enzyme (Hind III) digestion allows for amplification of fragments up to 2,000 bp [GeneChip 100K arrays, Affymetrix] [16,25]. The choice of restriction enzyme determines the sequence content of the reduced fraction of the genome [30]. Although these methods do not allow for producing the whole genome arrays, they are used in commercially available chips [Affymetrix] assigned for rapid sequencing of several genes linked with AIDS and cancers [17]. The GeneChip® HuSNP™ Array, commercially available from Affymetrix enables whole-genome surveys by simultaneously tracking nearly 1,500 SNP variations dispersed throughout human genome [16].

6. Microarray in the study of cardiovascular disease

Microarrays give the possibility to evaluate a wide spectrum of candidate genes and to simultaneously observe interaction of genes in an attempt to focus on specific culprits, on the identification of novel pathways associated with cardiovascular diseases for example and on the identification of therapeutic targets [31]. Coronary artery disease, a disorder influenced by either genetic or environmental factors, is a major cause of death. Genetic susceptibility to CHD and its complications is most probably conferred by relatively common allelic variations occurring in the population [32]. It is estimated that in the human heart, in any condition, about 25,000 of 35,000 genes may be expressed [33]. Microarray analysis of gene expression can be used to identify genes involved in disease progression and in disease reduction [33].

Chips also allow for the throughput and simultaneous analysis of a great variety of cell types such as cardiomyocytes, monocytes, macrophages, smooth muscle, endothelial, and fibroblasts and chemical mediators involved in cardiovascular disease pathology [34], their interactions and cumulative effects.

The first human cardiovascular cDNA microarray was constructed by Barrans and his collaborators [35]. They selected 38 genes involved in heart failure. Their analysis revealed 19 genes that were differentially expressed in failing compared to non-failing human hearts [36].

6.1. SNPs as genomic markers of CAD

SNPs are utilised as excellent genomic markers in linkage disequilibrium mapping or association analysis

as they are abundant, widespread [16] and represent the most popular form of human genetic variations causing or predisposing to diseases (e.g. Duchenne muscular dystrophy [37]).

It is estimated that about 1% (approximately 3,000,000) of nucleotides undergo variations in the human population [22]. Maps of SNPs are believed to help to identify multiple genes associated with multifactorial diseases such as cardiovascular disease and its risk factors (e.g. diabetes mellitus [38], high level of cholesterol [39]) as well as cancer, HIV, and mental illness. Contrary to well established biochemical and environmental risk factors of cardiovascular disease, the genetic risk alleles contributing to it in the general population are not well known [40]. Many experiments using microarray technique are designed with the aim of identifying the key genes in the development of this disease. Investigations of cases with premature CHD revealed the familial aggregation of this disease. Experiments conducted in Finland revealed that brothers of male CHD cases showed a 2.5-fold higher risk and sisters showed a two-fold higher risk [40]. Topol *et al.* [41] focused their research on genes associated with the development of familiar, premature cardiovascular disease and myocardial infarction. The study comprised 352 premature coronary artery disease cases and 418 controls recruited from a population of white Americans. Researchers examined the family of five genetically separate thrombospondin (TPS) genes using high-throughput microarray technology. Short oligonucleotides designed on the basis of thrombospondin genes, suspected of causing cardiovascular disease, were identical to a particular gene except for a single nucleotide. Probe sequences on a glass slide were hybridized with complementary targets obtained from individuals either positive for cardiovascular disease or negative (control group) [41-42]. Thombospondins, which are glycoproteins found in multiple organ systems, play an important role in blood clotting, wound healing, angiogenesis, tumor cell proliferation [42] and in complex cell-cell (intercellular) and cell-matrix (matricellular) interactions. Using these oligonucleotide arrays, Topol *et al.* [41] identified missense variations in the thrombospondin-1 (N700S) and 4 (A387P) genes that increase individual susceptibility to premature coronary artery disease and the mutation (T→G) in a non-coding region of a thrombospondin-2 gene that seems to be protective (in the homozygotic variant) against the development of heart disease. A missense variant (A387P) of thrombospondin-4, which is thought to affect folding and secretion of the protein and leads to the disruption of the calcium binding site, showed the strongest association. However, further research is needed to determine whether these genes are solely responsible for the disease or they only mark a functional mutation present in either this or a nearby gene [41]. Martin D. Tobin *et al.* [43] interrogated 63 SNPs in 35 candidate genes predisposing to myocardial infarction. They used sequence specific oligonucleotides attached to nylon membrane strips to analyse mutations in amplified DNA obtained from 549 Caucasian cases (aged <75 years) with myocardial infarction and 505 Caucasian controls. They found out that the adducin-1 (ADD-1) 460Trp allele has a protective effect against myocardial infarction. Polymorphism Gly460Trp in this cytoskeleton protein, involved in actin polymerization and cell signal transduction, influences sodium and water homeostasis. Also, functional mutation in a plasma cholesteryl ester transfer protein (CETP) is revealed to be protective, playing a key role in the metabolism of high density lipoproteins, by mediating the transfer of cholesterol esters from HDL to other lipoproteins, and in the uptake of cholesterol by the liver. Protective haplotype - CETP-629 is associated with reduced promoter activity and increased HDL levels, which is consistent with the common knowledge that a reduced amount of HDLs poses a strong atherosclerosis risk [43]. Martin D. Tobin *et al.* [43] also discovered variations in a family of tightly linked paraoxonase genes, which probably influence paraoxonase protein activity. The paraoxonases, located on the surface of HDL, are thought to play a crucial role in prophylaxis of atherosclerosis by preventing low density lipoprotein (LDL) oxidation. Polymorphisms 192Gln and 55Met in PON1 and Cys311 in PON2, associated with higher paraoxonase activity, lead to more efficient inhibition of LDL oxidation thus being protective against this disease [43]. Several studies confirm the protective effects of the above mentioned genes [44-45] while others find no association of PON mutations with the risk of coronary heart disease [46]. While analyzing polymorphisms at positions -641, -482, –455, -1100, 3175 and 3206, occurring in apolipoprotein C-III, Martin D. Tobin *et al.* [43] identified two haplotypes (CCTTCG and ATCCCG) associated with the increased risk of myocardial infarction. Some polymorphisms in genes such as lymhotoxin-α (Thr26Asn), PAI-1 (5G/4G and 5A/6A) and stromelysin-1, linked with myocardial infarction in different studies, show no association with heart disease in the Martin D. Tobin *et al.* [43] experiments.

T. Pastinen *et al.* [32] analyzed not only genes affecting the risk of MI but also their possible interactions in a genetically homogenous population of Finland. They applied minisequencing on primer arrays to study 12 common polymorphisms occurring within

8 genes, classically associated with CHD, such as: plasminogen activator inhibitor (PAI-1) and glycoprotein IIIa (GPIIIa), encoding proteins involved in fibrinolysis and platelet adhesion respectively, apolipoproteinn E (apoE), the coagulation factor XIII (FXIII), low density lipoprotein receptor - LDLR (four mutations), methylenetetrahydrofolate (MTHFR), angiotensin II type I receptor (AT1R) and angiotensinogen (ATG). Pastinen *et al.* [32] found the evidence of association of common variants of the glycoprotein IIIa (GPIIIa) gene and the plasminogen activator inhibitor (PAI-1) gene with MI in Finnish subjects. An individual's risk of MI was significantly increased in the concurrent presence of these two genetic variants, which does not seem to be surprising as both are involved in arterial thrombus formation. Insertion in the promoter region of the PAI gene affects the plasma levels of this protein while the GPIIIa genotype is found to be a significant factor predicting the thrombosis of coronary stents. Analysis of the rest of the genes failed to reveal any significant increase of CAD risk. Contrary to subsequent association studies, suggesting that the polymorphism in the FXIII gene confers protection against the development of MI, this study reveals no significant association [32]. Discrepancies between the results obtained in different studies may be due to the differences in the definition of disease phenotypes or in the choice of cases and controls, population heterogeneity and genetic diversity between the populations [32]. David A. Hinds *et al.* [47] reveal in their study that although most functional human genetic variations are not population-specific, they are shared across human populations with differences in allele frequencies [47]. Apart from gene polymorphisms, also infectious agents (e.g. Chlamydia pneumoniae, cytomegalovirus (CMV)) may contribute to the progression of atherosclerosis and the development of cardiovascular disease. The presence of viruses/bacteria may also be detected by PCR/microarray techniques [48].

Microarray analysis is also successfully used for identifying therapeutic targets. Jin and co-workers examined the effects of captopril on myocardial remodelling in rats and found 37 differently expressed genes [31]. The rising interest in the field of large-scaled SNP genotyping resulted in the creation of the International Haplotype Mapping Project, aiming at the determination of linkage disequilibrium patterns within the human genome that will facilitate selection of the most informative SNPs for association studies [49].

6.2. Expression studies of genes involved in CAD and MI

S.R. Archacki *et al.* [46], whose study aimed at the identification of genes differentially expressed in atherosclerotic human arteries, used chips with oligonucleotides representing approximately 12,000 unique genes. They examined samples obtained from nine severely atherosclerotic and six healthy human coronary arteries. The combination of two statistical tests generated a list of 401 differently expressed genes, 56 of which met the authors' criteria of genes associated with CAD. Fifty-five of these genes were up-regulated and only one down-regulated. Interestingly, 49 of these genes were not previously linked to CAD. S.R. Archacki *et al.* [46] classified differently expressed genes on the basis of their molecular activity into five groups: inflammation (genes encoding histocompatibility complexes MHC, immunoglobulins, complement component 2 and 4b, retinoic acid receptor responder, MIR-7, HEM45, CD37 antigen, T-cell receptor β-chain, lymphocyte-specific protein 1, human Ia invariant γ-chain, tapasin, 17 kDa/15 kDa interferon-stimulated protein, galectin-9, and tumor necrosis factor-α-induced protein-2); cell necrosis/apoptosis/proliferation (interferon stimulatory factor-3, XBP1, B144 NK cell triggering receptor-LST1, STAT-91, mitogen-responsive phosphoprotein -DOC-2, a hypothetical gene with homology to perlecan, arrestin-β2, platelet-derived endothelial cell growth factor -ECGF1, chemokine G-coupled receptor- fusin, B-cell activator gene -BL34, GOS8, fusin, Rho GTPase activating protein-4, and PIM-2); cell migration/adhesion and matrix degradation (lumican, VCAM-1 and its precursor, ICAM-2, osteopontin precursor, membrane alanine aminopeptidase- IGF1R, MMP-9, cathepsin H and K genes); lipid transfer/oxidation/metabolism (genes for steroidogenic acute regulatory protein, butyrophilin-BTF4, glutathione-S-transferase -GST, flavocytochrome 588, and chitotriosidase); and unspecified functions (expressed sequence tags -EST and two genes predicted to encode a small inducible cytokine and a homocysteine-inducible protein) [46]. This study, showing that immunoglobulins had the highest fold changes in expression, confirmed the hypotheses that inflammation plays a crucial role in the pathogenesis of CAD [46]. Conducted experimental results are in agreement with previous studies, claiming that increased expression of chitotriosidase, lumican, VCAM-1, ICAM-1, matrix metalloprotease-9 (MMP-9) and osteopontin precursor (OPN) occurs in case of atherosclerosis. However, the obtained results reveal the genetic profile of coronary arteries at a time point and do not inform us of the whole process of atherogenesis [46].

The list of candidate genes that have been linked to the risk of developing cardiovascular disease or coronary artery disease is constantly growing but due to discrepancies in these finding, there is a need to re-examine the obtained results or to seek more predictive haplotypes. Analysis of genetic markers predicting a disease and those predicting treatment response will be of great importance in coming years and will probably revolutionize the development of cardiovascular medicine.

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