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Review

Design issues in toxicogenomics using DNA microarray experiment

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Abstract

The methods of toxicogenomics might be classified into omics study (e.g., genomics, proteomics, and metabolomics) and population study focusing on risk assessment and gene–environment interaction. In omics study, microarray is the most popular approach. Genes falling into several categories (e.g., xenobiotics metabolism, cell cycle control, DNA repair etc.) can be selected up to 20,000 according to a priori hypothesis. The appropriate type of samples and species should be selected in advance. Multiple doses and varied exposure durations are suggested to identify those genes clearly linked to toxic response. Microarray experiments can be affected by numerous nuisance variables including experimental designs, sample extraction, type of scanners, etc. The number of slides might be determined from the magnitude and variance of expression change, false-positive rate, and desired power. Instead, pooling samples is an alternative. Online databases on chemicals with known exposure-disease outcomes and genetic information can aid the interpretation of the normalized results. Gene function can be inferred from microarray data analyzed by bioinformatics methods such as cluster analysis. The population study often adopts hospital-based or nested case-control design. Biases in subject selection and exposure assessment should be minimized, and confounding bias should also be controlled for in stratified or multiple regression analysis. Optimal sample sizes are dependent on the statistical test for gene-to-environment or gene-to-gene interaction. The design issues addressed in this mini-review are crucial in conducting toxicogenomics study. In addition, integrative approach of exposure assessment, epidemiology, and clinical trial is required.

Keywords: Toxicogenomics; Microarray; Study design; Epidemiology

Contents

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Introduction

Toxicogemomics is the evolving science which measures the global gene expression changes in biological samples exposed to toxic agents and investigates the complex interaction between the genetic variability and environmental exposures on toxicological effects (Olden et al., 2004). DNA microarrays have become most popular and important method to measure the expression of mRNA level offering great potential for environmental or toxicological studies. Gene expression changes can possibly provide more sensitive, immediate, comprehensive maker of toxicity than typical toxicological endpoints such as morphological changes, carcinogenicity, and reproductive toxicity (Marchant, 2003). The US National Institutes of Environmental Health Sciences Microarray Group has been using microarrays to analyze changing patterns of gene expression across the entire genome, studying thousands of affected genes at a time and revolutionizing the way that toxicologic problems are investigated (The National Center for Toxicogenomics, 2004).

Toxicogenomics has been defined as the genomic study in relation with exposure to toxic elements. Toxicogenomics can also be defined as the measurement of global gene expression changes in biological samples exposed to toxicants (Orphanides, 2003) or as the study of the response of the genome to toxic agent exposure (Marchant, 2003). In this regards, toxicogenomics includes genomic-scale mRNA expression (transcriptomics), cell and tissue-wide protein expression (proteomics), metabolite profiling (metabonomics), and bioinformatics. These studies can be grouped as "-omics" study, which could be applied to various kinds of samples and species.

The epidemiological population study focusing on genetic risk factors as well as environmental factors (i.e., exposure to toxic elements) can also be included in the category of toxicogenomics. Epidemiological study design has been emphasized as a basis for effective application of new technology such as genomics, proteomics, and metabolomics (Potter, 2003). Simmons and Portier (2002) have defined toxicogenomics as the application of knowledge of genes associated with disease states to the study of the toxicology of chemical and physical agents. In this context, the association studies evaluating the interactive effects between genetic factors (e.g., single nucleotide polymorphisms) and environmental exposure might be considered as one kind of toxicogenomics. Thus, the perspectives of epidemiological population study need to be supplemented or blended with those of "-omics" study.

In order to correctly interpret huge toxicogenomics data, a number of design issues need to be addressed. Thus, design issues in toxicogenomics using DNA microarray experiment are going to be discussed in this paper: from experimental objectives to the data analysis and interpretation.

Design issues of DNA microarray experiment

Experimental objectives

A study cannot be designed properly to meet the objectives until they can be clearly articulated before initiating the study (Page et al., 2003). The experimental objectives might be the generation of new objectives or hypotheses on interesting pathways or genes. However, investigators are encouraged to articulate in advance what they anticipate to get from microarray studies so that those studies can be designed appropriately from the beginning.

Selection of genes for microarray

It is important to determine how many and which genes should be measured to characterize a toxic response. Genes falling into several categories (e.g., xenobiotics metabolism, DNA repair, regulation of cell division, cell signaling, cell structure, apoptosis, metabolism etc.) can be selected according to a priori hypothesis and known mechanism through which a toxicant is working (Pennie et al., 2000; Smith, 2001).

Pennie et al. (2000) constructed human and mouse ToxBlot arrays including gene classes related with cancer, immunology, endocrinology and neurobiology, investigative toxicology, predevelopment toxicology, and safety assessment. ToxBlot arrays were composed of approximately 2400 cDNA sequences, spanning about 600 genes of the relevant species. Four individual spots containing two non-overlapping cDNAs on each array represented one gene.

When selecting genes for the knowledge-based microarrays, several online resources for biological data and information for toxicogenomics study including GeneCards (Rebhan et al., 1997), KEGG, NTC, TRC, CEBS etc. can be referred to and a substantial matrix of data on chemicals with known exposure-disease outcomes need to be obtained (Table 1).

Table 1
Online resources for biological data and information for toxicogenomics study

Source	Link
TOXNET	http://toxnet.nlm.nih.gov/
NIEHS NCT (National Center for Toxicology)	http://www.niehs.nih.gov/nct/
TRC (Toxicogenomics Research Consortium)	http://www.niehs.nih.gov/nct/trc.htm
CEBS (Chemical Effects in Biological Systems)	http://www.niehs.nih.gov/nct/cebs.htm
NIEHS Microarray Group	http://dir.niehs.nih.gov/microarray/home.htm
MGED (Microarray Gene Expression Data Society)	http://www.mged.org/
EBI (European Bioinformatics Institute)	http://www.ebi.ac.uk/Information/sitemap.html
GeneCards (Weizmann Institute)	http://bioinfo.weizmann.ac.il/cards/index.html
KEGG (Kyoto Encyclopedia of Genes and Genomes)	http://www.genome.ad.jp/kegg

Selection of microarray platform

Brief diagram of procedures of DNA microarray is shown in Fig. 1. In cDNA microarray, total RNA or mRNA is isolated from control and treated tissues and reverse transcribed in the presence of radioactive or fluorescent labeled nucleotides, and the labeled probes are then hybridized to the arrays. The intensity of the array signal is measured for each gene transcript by laser scanning confocal microscopy. The ratio between the signals of control and treated samples reflects the relative toxin or drug-induced change in transcript abundance. Current microarray technology allows the simultaneous expression monitoring of 20,000–25,000 genes.

Microarrays platforms can be divided into three categories: spotted cDNA, Affymetrix arrays, and spotted oligonucleotide arrays (The Tumor Analysis Best Practices Working Group, 2004). In a cDNA microarray, each gene of interest is represented by a long DNA fragment (200–2400 bp) typically generated by polymerase chain reaction (PCR) and spotted on glass slides using robotics (i.e., pin or inkjet method). In Affymetrix arrays, the probes are short oligonucleotides (15–25 bp) synthesized directly onto a solid support using photolabile nucleotide chemistry. And, spotted oligonucleotide arrays were recently developed using synthetic oligonucleotides (30–100 bp). The advantages and disadvantages of cDNA and oligonucleotide microarray are compared in Table 2.

Another option is to choose an appropriate microarray platform (slide) developed for toxicogenomics study by commercial vendors (e.g., Affymetrix, Nanogen, Amersham, Nimblegen, Phalanx, Agilent, Incyte, Hyseq etc.). For the

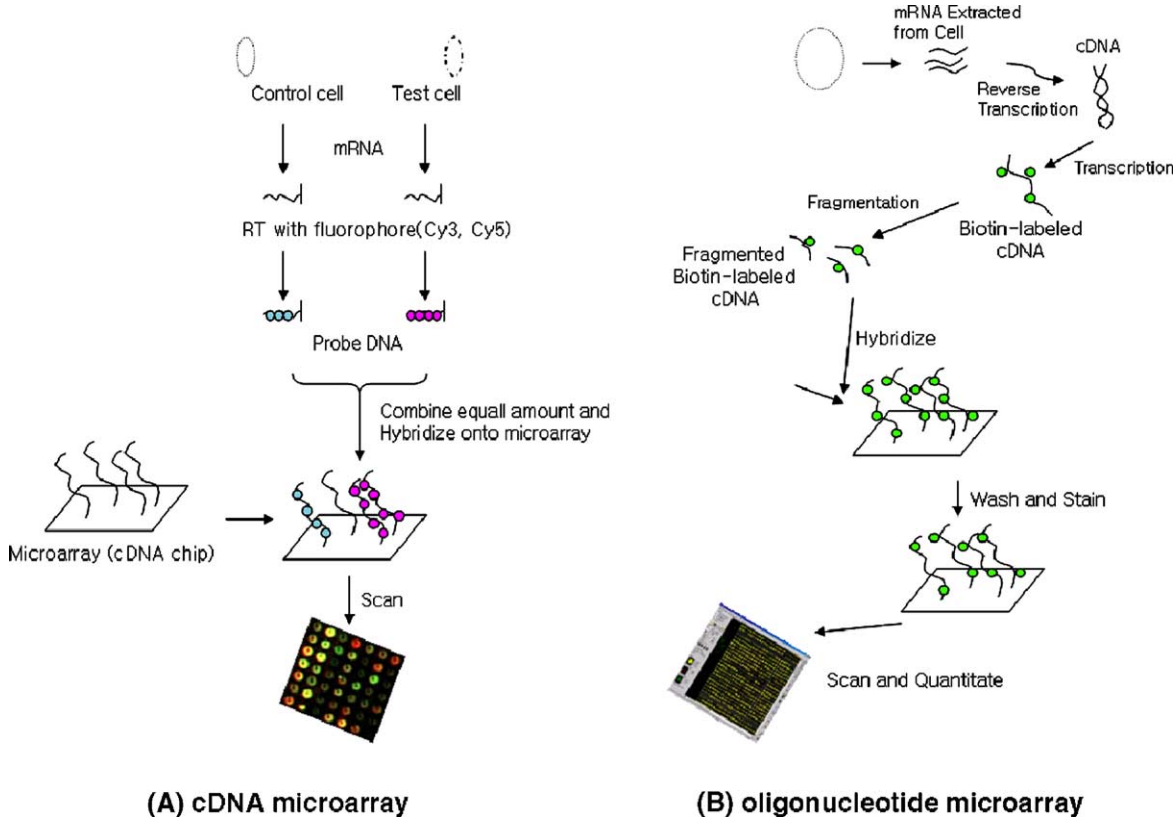

Fig. 1. Overview of the procedures of DNA microarray (cDNA and oligonucleotide).

Table 2
Comparison of cDNA arrays and oligonucleotide arrays

	Advantages	Disadvantages
cDNA arrays	The content of each microarray is determined by the researcher	Variable amount of DNA in each spot
	The cost per array is relatively low	Specificity of the hybridization to the relatively large cDNA inserts
	Proper for comparison of global gene expression between different environmental exposure	
Oligonucleotides arrays (Affymetrix GeneChip)	Synthesized probe is typically of known concentration, of known sequence	Relatively high cost of synthesizing large numbers of large oligonucleotides
	Most of the process can be automated, leading to less samples mix-up and less drop-out of samples	Selection of small sequences of whole gene is problematic
	Proper for experiment related with single nucleotide polymorphism	Non-renewable nature of the resource

purpose of comparison in global gene expression among the samples with different exposure or treatment, as is often the case in toxicogenomics, cDNA microarray has been preferred. However, the oligonucleotide microarray becomes more and more popular as the cost, which was the major obstacle, becomes substantially lower recently.

Design of DNA microarray

A number of variables contribute to experimental variability. The possible sources of variation are the variation among the experimental units (e.g., rats), tissue extraction, mRNA extraction, cDNA preparation and labeling, hybridization, washing, reading, DNA spot, and between-array variation, etc. Microarray experiments can be affected by numerous nuisance variables which are related to experimental designs, sample extraction, type of scanners, etc. Quality of matrix (i.e., yield, reliability, and validity etc.) can be influenced by the type of samples (peripheral blood, animal tissues, etc.), the purity of samples, collection, shipping, and storage (Page et al., 2003). Intra-subject, inter-subject, inter-group, and technical variation (microarray protocol) are related with overall design of microarray experiment and statistical power.

The Minimum Information About a Microarray Experiment (MIAME) developed by the Microarray Gene Expression Data Society (MGED: http://www.mged.org/) is the guidelines for microarray data and reporting them (Brazma et al., 2001). The MIAME guidelines include descriptions of experimental design (e.g., number of replicates, nature of biological variables) and experimental procedures (e.g., sample type, extraction, and hybridization).

The design issues of DNA microarray experiment were categorized into five areas: experimental design, species and sample types, replicates, sample size, and data analysis and interpretation.

Experimental design

The first and most important step of designing DNA microarray is to determine which mRNAs are to be labeled with which fluorescence dye and which are to be hybridized together on the same slide. However, there are several constraints for selecting proper design: the number of slides, the amount of RNA available, and cost.

Examples of different designs for DNA microarray experiments are illustrated in Fig. 2. Direct design becomes unlikely to be feasible or desirable for a large number of comparisons because of the limitation of the amount of mRNA and cost, whereas indirect reference design becomes more popular and is by far the most widely used. Alternative class of designs is loop design (Kerr and Churchill, 2001), in which the graph forms a single loop that connects successive pairs of vertices.

Multiple doses are essential component for the detection of dose-related effects such as threshold in toxicity and dose–response relationship. Time series is also useful to understand the biochemical processes associated with chronic chemical exposure.

Species and sample types

The appropriate species and type of samples should be selected to maximize the likelihood of true positives and minimize false-negatives. Key variables include tissue heterogeneity, stage of disease, and inter-individual variation, all of which have been found to be major confounding variables (The Tumor Analysis Best Practices Working Group, 2004).

Ezendam et al. (2004) found varied gene expression profiles in various organs of Brown Norway rats exposed to hexachlorobenzene: spleen, mesenteric lymph nodes, thymus, blood, liver, and kidney. It is important issue to determine whether or not serum and/or blood cells can be used as informative subset to specific target organ tissue (The National Center for Toxicogenomics, 2002).

Considerable inter-species variability of cytochrome *P*405 2E1 (CYP2E1) activity was observed between rodents and non-rodents (rabbit and human) (Bernauer et al., 2000), whereas intra-species or intra-strain variability in rodents was small (Bernauer et al., 1999). Moreover, inter-individual variability is expected to be larger in outbred (genet-

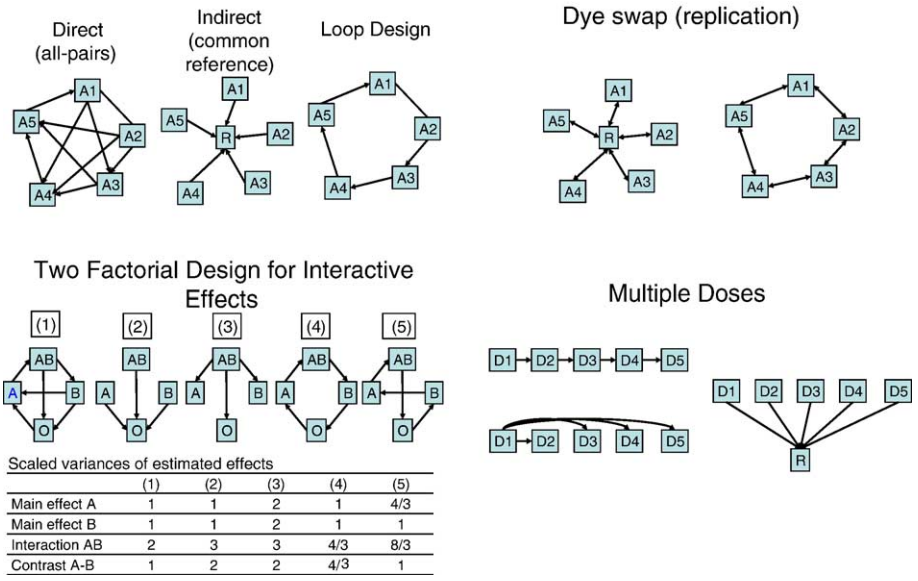

Fig. 2. Experimental design of cDNA microarray (allocation of mRNA samples to the slides).

ically heterogeneous) humans than that among inbred mice (Wei et al., 2004).

Replicates

Replicates reduce variability in summary statistics and in turn the rate of false-positives (Yang and Speed, 2002). However, there is no gold standard about the number of microarray replicates since it is dependent on biological variability in the study samples and time point or multiple doses of experimental design.

The type of replication used in a given experiment affects the precision and the generalizability of the experimental results. One common form of replication is putting replicates of the same spot (cDNA probe) on each slide (Black and Doerge, 2002). In general, less variation is observed within slides than between slides. Data from replicate spots are valuable for monitoring and improving the overall quality of the experimental data, but adjacent spots need to be avoided. Nearly all aspects of the experiment such as printing, general hybridization, and scanning conditions will be shared by adjacent spots, thus systematic errors could not be monitored.

Replicates between slides have been divided into technical and biological replicate (Quackenbush, 2002). Technical replicate between slides refers to replication in which the target mRNA is from the same extraction or pool, therefore resulting in a smaller degree of variation in measurements. Biological replicate refers to hybridizations that involve mRNA from different extractions, e.g., from different samples of cells from a particular cell line or tissue. This type of replication has sometimes been considered as sample size, i.e., the number of samples or slides in each treated group.

Lee et al. (2000) recommended that at least three (technical) replicates be used in designing experiments by using cDNA microarrays, particularly when gene expression data from single specimens are being analyzed. It is advisable to have replicates well spaced and not adjacent, as this would give a better reflection of the variability across the slide. Replication is closely connected with the statistical extrapolation from sample to population.

Dye swap (dye-flip) replications involve two hybridizations for two mRNA samples from the same extraction, in which dye assignment is reversed in the second hybridization (Fig. 2). This type of replications is useful for reducing systematic bias.

Sample sizes

In microarray experiment, the definition of sample size is somewhat confusing. Sample sizes could be defined as several slightly different meanings: i.e., number of biological replication, total number of slides, or total number of individuals.

Although several studies have reported the sample size calculation methods in microarray experiment (Black and Doerge, 2002; Hwang et al., 2003; Pan et al., 2002; Wei et al., 2004; Zien et al., 2003), it is a very complicated process. A sample size calculation includes at least four components: (1) the variance of individual measurements, (2) the magnitude of the effect to be detected, (3) the acceptable false-positive rate, and (4) the desired power, that is, the probability of detecting an effect of the specified (or greater) magnitude. The first two components can be obtained from the data of previous and pilot studies (Yang and Speed, 2002). Large false-positive rate will occur as a result of multiple tests, even with small (e.g., 5%) chance of being false-positive in each test. Therefore, several methods such as Bonferroni correction are used to adjust false-positive rate considering the objectives of the study. When mRNA samples for the experiments are scarce and the verification

method is straightforward and relatively cheap, a higher false-positive rate is tolerable.

Practically, up to ten inbred mice per group are required for treatment with toxic agent (Ezendam et al., 2004; Satomi et al., 2004), whereas human samples require considerably more individuals per group classified by exposure status (Lampe et al., 2004). Longitudinal design with varied duration (time) of exposure makes the required number of (biological) replicates in each treatment group smaller. Time points can be selected based on preliminary studies. Multiple doses which are suggested to identify those genes clearly linked to toxic response can also reduce number of (biological) replicates in each treatment group. On the other hand, as the number of time points or doses increases, the total number of slides increases (Fig. 2).

Tissue pooling from many individuals in the same treatment group is another alternative (Churchil, 2002; Peng et al., 2003). The adoption of pooling strategy is mainly due to the quantity of samples and the cost. This approach also seems to be appropriate in a large population epidemiological study with microarray experiment. When pooling the samples in population study, careful epidemiological concepts should be considered such as matching, randomization, and misclassification of exposure status, which are briefly described in the section of population study.

Data analysis and interpretation

In cDNA microarray experiments, a pair of images is produced and processed by different kinds of software for image analysis to (R,G) fluorescence intensity pairs for each gene on each array (where R = red for Cy5 and G = green for Cy3). An 'MA-plot' is used to represent the (R,G) data, where $M = \log_2 R / G$ and $A = \log_2(R \times G)^{1/2}$. This is useful for identifying spot artifacts and detecting intensity-dependent patterns in the log ratio M and normalization procedures as well.

Normalization refers to the process of removing systematic variation in microarray data (Yang and Speed, 2002). Normalization usually consisted of several procedures such as global normalization which assumes that the red and green intensities are related by a constant factor, intensity-dependent normalization, within-print tip group normalization, and between slide normalization. When dye swap replication was used in cDNA microarray, then self-normalization is conducted under the assumption that the normalization functions are the same for the two slides.

After removing or minimizing the systematic variations with normalization, one obtains gene expression matrices tables where rows represent genes, columns represent various samples such as tissues or experimental conditions (Brazma and Vilo, 2001).

If two rows are similar, the respective two genes are coregulated and possibly functionally related. Gene function can be inferred by various bioinformatics approaches including clustering, classification, and pattern discovery. These analyses are usually represented by dendrogram (Fig. 3).

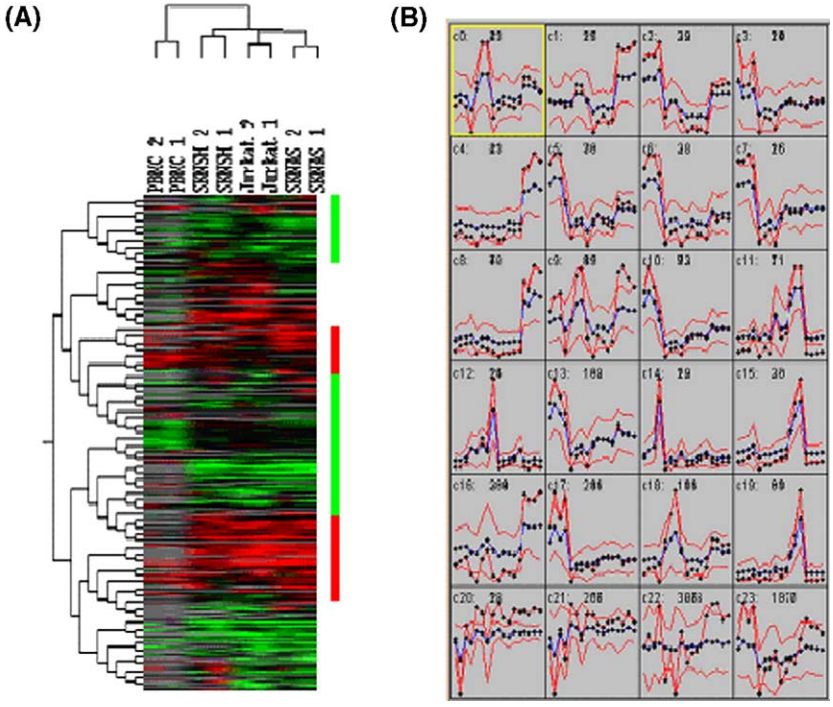

Fig. 3. Cluster analysis and graphical display of genome-wide expression patterns (Jurkat T cells under gamma irradiation). (A) Hierarchical clustering creates functional clusters with color-coded expression patterns. (B) Partitional clusters with genomic grid structure are created by self organizing maps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Treatment effect can be determined by comparing columns by which genes are differentially expressed according to different treatments. This is related with hypothesis testing by multivariate analysis for the experiment with specific design. The computational statistics like permutations are often used because the normality assumption often could not be assumed.

Design issues in epidemiological population study

One of the major challenges in toxicology is how the effects observed in vitro models or in experimental animals can be properly related to the probable effects in humans for risk assessment and regulatory decisions (Smith, 2001). This is why the epidemiological population studies are needed for toxicogenomics study.

Epidemiology is an observational science that describes the patterns of diseases and their determinants in human populations. Because the study groups in epidemiology classified by disease status or exposure status cannot be treated as experimental units, comparability between groups might not be ensured easily. Therefore, study design issues are central to good epidemiological practice and ultimately to the use of new technologies such as microarrays (Potter, 2003).

Bias and confounding

Selection bias resulting from the failure to collect all or a well-defined random subsets or cases of interest could be the most cumbersome problem in population epidemiologic study. This kind of bias arises from the way that study participants are selected from the source population. If selection bias cannot be avoided or controlled, then it may still be possible to assess its likely strength and direction of effects on the association between exposure and outcome.

Information bias may occur when there is misclassification of exposure or disease. If misclassification of exposure (or disease) is unrelated to disease (or exposure), then the misclassification is non-differential. If misclassification of exposure (or disease) is related to disease (or exposure), then the misclassification is differential. Exposure assessment without misclassification bias gaining a better estimate of exposure and internal dose is an important direction and challenge in future research of toxicogenomics.

Confounding addresses the distortion of association between an exposure and disease risk due to an extraneous factor that (1) is a risk factor for the disease, (2) is associated with the exposure, and (3) is not an intermediate step in the causal pathway between the exposure of interest and disease (Rothman and Greenland, 1998). Confounding occurs when the exposed and non-exposed groups in the source population are not comparable because of inherent differences in background disease risk. The method to prevent confounding in advance of data analysis in population study is randomization and matching the potential confounding variables such as age, sex, etc. Confounding bias should also be controlled for in stratified or multiple regression analysis after collecting data. If there is the potential for uncontrolled confounding, then it is important to attempt to assess its likely strength and direction.

Sample size

A number of population studies were conducted to evaluate the effect of genetic variant on specific diseases. These population studies tend to be evolved into large genomic cohort or cohort consortium focusing on gene-to-environment or gene-to-gene interactions. Nested case-control study is the appropriate design for this cohort-based population study. In this case, optimal sample sizes are dependent on the statistical test for gene-to-environment or gene-to-gene interaction rather than for main effect of genetic variant or environmental exposure (Gauderman, 2002a, 2002b) (Table 3). As shown in Table 3, several thousands of cases need to be recruited for the evaluation of multiplicative or additive interactions given relatively small prevalence of exposure and genetic risk factor.

Therefore, pooling samples might be one of good choice in the application of "-omics" study to population study in condition that epidemiologically sound strategies to control

Table 3
Sample size estimation based on multiplicative gene–environment interaction in nested case-control study

P_G	P_E	R_G	R_E	P_D (%)	$R_{interaction}$	Case (N1)	Control (N2)
0.50	0.50	1.5	1.5	5	1.5	3340	3340
0.30	0.30	1.5	1.5	5	1.5	3746	3746
0.20	0.20	1.5	1.5	5	1.5	5799	5799
0.15	0.15	1.5	1.5	5	1.5	8682	8682
0.10	0.10	1.5	1.5	5	1.5	16,592	16,592
0.20	0.20	1.8	1.8	5	1.5	5769	5769
0.20	0.20	1.5	1.5	5	1.5	5799	5799
0.20	0.20	1.4	1.4	5	1.5	5847	5847
0.20	0.20	1.3	1.3	5	1.5	5921	5921
0.20	0.20	1.2	1.2	5	1.5	6032	6032
0.20	0.20	1.5	1.5	3	1.5	5696	5696
0.20	0.20	1.5	1.5	5	1.5	5799	5799
0.20	0.20	1.5	1.5	7	1.5	5904	5904
0.20	0.20	1.5	1.5	10	1.5	6063	6063
0.20	0.20	1.5	1.5	12	1.5	6170	6170
0.20	0.20	1.5	1.5	5	2.0	1950	1950
0.20	0.20	1.5	1.5	5	1.8	2725	2725
0.20	0.20	1.5	1.5	5	1.6	4294	4294
0.20	0.20	1.5	1.5	5	1.5	5799	5799
0.20	0.20	1.5	1.5	5	1.4	8474	8474
0.20	0.20	1.5	1.5	5	1.3	14,045	14,045
0.15	0.10	1.5	1.5	5	2.0	3943	3943
0.15	0.10	1.5	1.5	5	1.8	5556	5556
0.15	0.10	1.5	1.5	5	1.7	6872	6872
0.15	0.10	1.5	1.5	5	1.6	8838	8838
0.15	0.10	1.5	1.5	5	1.5	11,996	11,996

P_E: prevalence of environmental factor; P_G: prevalence of genetic factor; P_D: prevalence of disease in population (calculated by QUANTO Version 0.4.2 (Beta)).

potential confounders such as randomization and matching are adopted (Churchil, 2002; Peng et al., 2003).

Conclusion

In this review, we extended the definition of toxicogenomics to include the epidemiological population study evaluating gene–environment interaction. Epidemiological study design serves as a basis for effective application of a new technology of microarray (Potter, 2003). For example, Lampe et al. (2004), with a case-control design, showed that active exposure to tobacco smoke is associated with a biologically relevant mRNA expression signature in human population.

On the other hand, to minimize false discovery rate in toxicogenomics, the database of baseline gene expression in human samples needs to be constructed (Waters et al., 2003). Baseline gene expression among individuals is expected to vary widely with differences in age, nutritional status, developmental stage, personal habits, and health status, thus, changes due to environmental chemical exposure may not be greater than the noise of gene expression variability.

With this knowledge base, a number of design issues in microarray experiment addressed in this review are crucial to get more valid data from toxicogenomics study. Furthermore, an integrated approach of exposure assessment, epidemiology, and clinical trial will allow toxicogenomics to quickly identify the exposure-related susceptibility genes and characterize their functions in human cells (Olden et al., 2004; Smith, 2001).

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