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Combining genome-wide and targeted gene expression profiling in drug discovery: microarrays and real-time PCR

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Genome-wide examination of transcriptional events involved in drug action can provide new foundations for more mechanistic-based studies of the molecular and physiological basis of the effects and disposition of therapeutic agents. Although microarrays offer the allure of assessing gene expression levels for thousands of genes simultaneously, this technology is inherently exploratory in nature and interpretation of the results can be overwhelming and ambiguous. The statistical methods necessary to assign significance for array data have yet to be standardized or agreed upon by researchers. Here, one developing field of analysis using permutation-based procedures is reviewed. Quantitative real-time PCR, a fast and relatively inexpensive technique, can then be used to validate and extend array findings.

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▼ Genomic technologies such as cDNA or oligonucleotide microarrays and quantitative reverse-transcription PCR (QRT–PCR) are quickly becoming standard exploratory tools in drug discovery, drug development and clinical trials. Unlike previous technologies, these genomic techniques, particularly microarrays, yield a bewildering amount of noisy data. The analysis and interpretation of the results from these new technologies is complicated by the lack of standardization of both the collection of these data and the statistical procedures that are used to assess the significance of observed gene expression patterns. The interpretation of microarray data is further complicated by our incomplete knowledge of the complexity of biological systems (e.g. the multitude of possible gene interactions) and our lack of a comprehensive understanding of the organization of life at the molecular level (e.g. the relationship between mRNA levels and protein abundance). What is exciting about these technologies is that it is now possible to collect the data necessary to begin the examination of the complexity of life at the genetic and molecular level.

This review presents one strategy to conduct pharmacogenomic studies. A two-tier approach is used. The first level uses one set of flexible statistical tools (permutation analyses) to identify a list of significantly altered genes from microarray experiments. In the second step, real-time QRT–PCR is used to verify and extend the analysis of these new gene targets for drug discovery and development.

Microarrays and drug discovery

DNA microarrays [1–3] are now widely available from several commercial vendors and university core facilities, or can be fabricated on site. Densities of gene targets on a single array can range from tens to well over ten thousand cDNAs or oligonucleotides, providing an estimate of gene expression ranging from nearly the entire transcriptome to smaller pathway-specific groups of genes. The hybridization of labeled cDNAs or RNAs to the arrays is relatively straightforward and can be carried out in most laboratories with minimal equipment. The methodologies used in the analysis of microarray data largely reflect early microarrays that consisted of PCR-derived gene targets printed on prepared glass slides. These arrays are probed simultaneously with labeled cDNAs reverse-transcribed from

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two different mRNA pools (e.g. treatment and control or two different time points). Using different fluorescent dyes for each mRNA pool on the same array provides a straightforward method to control for experimental errors associated with the hybridization process. Results of these array experiments consist of image files containing intensity values (minus some assessment of background) resulting from the hybridization of labeled cDNAs to spots (genes) on the array. Before any comparative analysis of the data spots, intensities are typically normalized on each array to remove errors associated with experimental methodologies, such as starting RNA quality or quantity, differences in reverse-transcription efficiencies and labeling and hybridization kinetics [4–6]. Normalization methods vary [7–10], but the basic aim is to remove bias within each array by normalizing spot intensities to some subset of spots on the array ranging from all or nearly all spots (global normalization) to identified 'house-keeping genes' or control spots. Traditionally, data for each gene are reported as a ratio (often $\log_2$) of the two signals, with some arbitrary threshold set as indicative of a significant change in gene expression. This paradigm for array analysis has become standard even though many arrays, if not most arrays that are currently being used, do not allow for the hybridization of two labeled probes to a single array. Rather, many microarray experiments use a single array for each experimental treatment; thus there is no commonality between any two samples and therefore no rationale for calculating ratios before statistical analysis. More importantly, this paradigm of array analysis has continued even though many experimental designs do not lend themselves easily to two-sample comparative approaches. Recently, many researchers have pointed out the crucial need for appropriate experimental designs and the application of classical statistical techniques [11–15].

Microarray analyses: significant gene identification or data-mining

There exists a large and rapidly growing body of literature dealing with statistical issues regarding microarrays, with several excellent reviews of general statistical methodologies [11,16–22]. The statistical methods applied to microarray analyses can be divided into two broad classifications reflecting two different desired outcomes. The first group of analyses concerns the identification of a subset of genes from the many thousands assayed in an experiment that exhibit significantly altered gene expression because of the experimental conditions. The second broad group of analyses, which are often referred to as 'data-mining', use intensive computational methods to identify patterns of gene expression. Within this second group there are generally two different types of analysis representing distinctly different outcomes. In many cases, the goal is to classify or cluster genes into groups showing similar patterns of expression. Because these techniques do not provide any additional information to the computational algorithm other than the normalized intensities values, they are referred to as 'unsupervised approaches'. Examples include cluster analysis [23], *k*-means clustering [24] and self-organizing maps [25]. In contrast to these clustering methods, researchers often seek to group samples into functional classes (e.g. diseased versus normal tissues) based on gene expression patterns. The ultimate goal is to identify a subset of genes that will enable the researcher to make predictions or assignments of unknown samples based on gene expression patterns. Because the algorithms are provided with intensity data, as well as additional data in terms of classification groups, such methods are referred to as 'supervised approaches' [26].

Identifying significant genes

We will consider only the first level of analysis; that is, identifying those genes that exhibit significant differences in gene expression. Often this analysis must be completed before mining the data for patterns. The goal is to produce a list of genes where expression differs significantly among two or more sets of samples. In most cases, it is preferable to base decisions concerning membership in this 'significant' gene list on criteria other than some arbitrary threshold change in gene expression. In addition to the lack of statistical rigor [27], simple comparisons of fold change unfortunately mask information concerning the absolute levels of gene expression and limit the number of statistical tests available to assess the level of confidence in the inclusion of a particular gene in the significant gene list. There is a large number of parametric and nonparametric [28–34] tests available to assess gene significance if actual spot intensities rather than ratios are used in the analysis. The more pernicious problem with the statistical analysis of microarray data is the issue of multiple testing. Problems arise when any statistical test is used to evaluate the significance of a change in gene expression for hundreds to thousands of genes. For example, a statistical test to evaluate a significant change in gene expression at a P value of 0.05 might falsely reject the null hypothesis (i.e. no change in gene expression) for 250 genes from an array experiment assaying 5000 genes. As the number of hypotheses tested increases, the probability of rejecting a true null hypothesis (type 1 error) increases (Figure 1). This is not a new problem in statistics [35,36]; however, the massive number of genes tested in a typical array experiment makes this problem completely unavoidable. Standard methods to

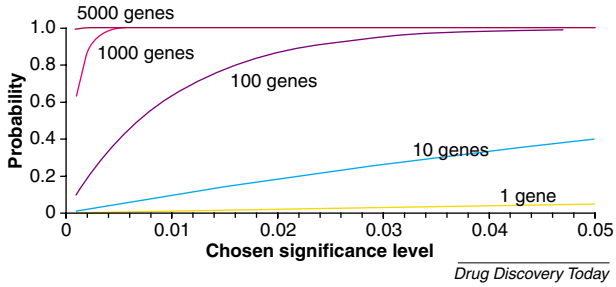

Figure 1. The multiple tests problem. The probability of obtaining a significant result by chance alone as a function of the number of genes examined in a microarray experiment and chosen significance level. With most arrays having a minimum of 100–1000 gene probes, the probability of finding a significant gene in any experiment is nearly assured, even at traditionally stringent α-levels of 0.01.

deal with the multiple-testing problem include procedures such as the Bonferroni adjustment. In its simplest form, this procedure multiplies the nominal *P* value obtained from each treatment by the number of independent tests being conducted. This solution is less than ideal for microarray analysis because the adjustment in significance levels comes at a cost in terms of a high loss of statistical power; that is, adjustments such as the Bonferroni that seek to control the family-wise error rate (FWER, Box 1) greatly increase the probability of committing a type 2 error (accepting a truly false null hypothesis). Thus, in seeking to obtain a list of genes that are all significant with the probability of 0.05, several genes that are most certainly significant will be removed from the list. Because microarray experiments are exploratory in nature, it is probably more desirable to include a number of false positives rather than discard some truly significant events. In addition, the Bonferroni procedure assumes that the multiple tests are independent of one another. This assumption is probably invalid given the known interdependence of many gene pathways.

Here, the statistical methods using permutation-based procedures to assess significance in microarray experiments are reviewed. These resampling or bootstrapping approaches are a significant addition to traditional statistical methods that typically control the overall type 1 error rate, such as the Bonferroni, and are appropriate for such massive and correlated datasets. Permutation-based procedures or bootstrapping techniques have been used extensively in other fields of biology (evolutionary biology, phylogenetics and population genetics [37]) and have wide application at all levels of microarray analysis. These techniques allow for the estimation and control of the false discovery rate (FDR, Box 1) [38–43] in assessing the significance of individual genes.

Box 1. Family-wise error rates versus false discovery rates

Family-wise error rate (FWER)
FWER seeks to control the probability of erroneously accepting an outcome as significant that is truly not significant (type 1 error). When conducted multiple times, the probability threshold indicates the chance of committing even one type 1 error. All statistical procedures that control FWER yield a list of genes in which it can be assumed with confidence that at the given probability level there are no false positives. This is achieved at the cost of excluding some genes that might actually be significant. In many cases in array experiments, researchers are willing to allow for the inclusion of a few false positives rather than exclude genes that are truly significantly altered in their expression.

False discovery rate (FDR)
FDR procedures seek to control the proportion of false positives among the list of genes identified as being significantly altered in expression. FDR is distinct from the false positive rate, which is the proportion of truly non-significant outcomes identified as significant. Unlike FWER, FDR is calculated after the list of significant genes has been generated. Generally, the FDR is estimated using the gene permutations of the gene expression data to determine the number of false positives.

Permutation analysis

Among the many methods of analyzing microarray data are a class of nonparametric statistical techniques that are based on permutation analysis. The basic methodology consists of comparing a chosen test statistic calculated from the data against a null distribution of the same text statistic constructed using random permutations of the data itself. This methodology has several advantages over standard parametric procedures in that these methods do not require the usual assumptions of a parametric test and such permutation-based tests take advantage of the large, often poorly replicated, microarray datasets. Briefly, the dataset is reordered randomly to create a 'new' dataset from which a new test statistic can be calculated (Figure 2). For microarray experiments, this is generally accomplished by reordering the columns of data where each column represents either treatment or control, or some different time point (different arrays). A single column would consist of the intensity values for each gene on the array and is thus anywhere from hundreds to thousands of entries in length. In reordering the columns to construct the permutation dataset, the order of the genes

is maintained and therefore the dependency of the genes with one another. This process of permutation can be repeated as many times as there are unique permutations. For datasets with limited replication, the number of unique permutations with which to build the null distribution is small. For example, for the example shown in Figure 2, there are 70 possible permutations, 35 of which are unique; the other 35 permutations are simply the inverse of the first 35. The null distribution that can be constructed using these data would have only 35 values. This is often the major limitation to this methodology [42,44]. This simple procedure was employed to identify a list of significant genes that had been altered in expression as a result of nitrate exposure using an array with 5531 genes [45] and a second pathway-specific array with only 100 genes [46]. In the larger study, there were four treatment samples and four controls. Standard *t*-tests with unequal variance identified 447 genes that were altered in their expression by the nitrate treatment. Given a *P* value of 0.05, it could be expected that there would be 276 significant outcomes by chance alone (type 1 errors) when conducting a statistical test 5531 times. We used the 35 unique permutations to build null distributions for each gene. On average, each permutation of the data yielded 149 false positives; that is, genes that were 'significant' at the $P < 0.05$ level using randomized data. Using the simple criteria of accepting as significant any gene for which the *t*-statistic was significant at $P < 0.05$ and for which the *t*-statistic was at the tail of the constructed null distribution (largest *t*-value among the 35 permuted *t*-values for each gene), we reduced the list of significant genes (as identified by traditional *t*-tests) to 290. Thus, for the 447 genes originally identified as significant, 157 (447 – 290 = 157) yielded higher *t*-values using randomized data. These genes were removed from the list of significant genes as probable false positives. Westfall and Young [47] have used permutation-based procedures combined with 'Bonferroni-like' step-down adjustments to control the FWER for multiple testing, and propose that the procedure takes into account the dependence between genes. This technique yields significance values with the traditional interpretation; that is, the probability of committing at least one type 1 error in the entire dataset does not increase with the number of tests. However, the Westfall and Young procedure is conservative. When applied to our data above, the list of significant genes is reduced to 80. However, this strong control of the family-wise type 1 error rate, results in increased type 2 errors. Among the 367 genes (447 – 80 = 367) now removed from the list of significant genes, there is a high probability of some being significant.

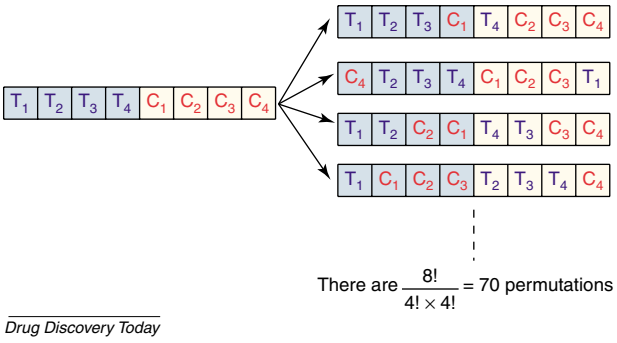

Figure 2. One method of generating permutations of a microarray dataset. In this example, there are four treatment arrays (T_1–T_4) and four control arrays (C_1–C_4). Each array represents a column of intensity values for each of the genes on the array. Permutations are built by randomly reassigning arrays into treatment and control groups. For each permutation, a new set of test statistics is calculated to build a distribution of values with which to compare the 'real' test statistic.

One final permutation-based statistical procedure for which the software is now readily available is the statistical analysis of microarrays (SAM [44,48]). SAM is based on permutations of the dataset using a modified *t*-statistic (a regularizing constant is added to the denominator of the *t*-statistic to remove biases associated with small variances). Columns of data are reordered randomly with each permutation to yield a distribution of *t*-values from which an 'expected' *t*-value can be calculated to compare with the 'real' *t*-value for each gene. SAM outputs a list of significant genes based on user-defined rejection rules. SAM also estimates the FDR when the specified rejection rules are inputted. The user can then adjust the rejection rules to increase or decrease the FDR (and the list of significant genes) depending on the objectives of the study. This is one of the major advantages of permutation-based procedures. The ability to estimate and adjust the FDR allows researchers to assess significant gene lists given different tolerance levels for false positives. Often the inclusion of some small number of false positives might be far less detrimental to an analysis where the goal is to identify gene expression patterns than it is to remove some truly significant genes to control the FWER.

Comparisons among permutation-based methods, parametric tests and traditional nonparametric tests indicate good concordance [30,42,45,49]. Although permutation-based tests are nonparametric, such methods are only as good as the null statistics constructed using the permutations. Improvements on permutation-based methods have recently been proposed to improve the fit between test and null distributions [31,32,50]. One of the major limitations

of permutation-based methodologies is the granularity of *P* values that arises when the number of permutations is small because of limited sample size. This results in analyses for which the number of genes identified as significant changes sharply with small changes in the FDR. This is a particular problem when the number of samples in each group is less than five.

Permutation-based procedures enable researchers to assess the robustness of the conclusions by increasing or decreasing the FDR. Finally, permutation-based methods are extremely adaptable. Virtually any statistic, including clustering algorithms and other data-mining procedures, can be tested using random permutations of the data.

Quantitative real-time PCR: validating and extending microarray results

Microarray experiments by their nature are exploratory exercises. The extreme complexity of the processes of life, combined with the many sources of error associated with the microarray technology itself, yield data that, even under the most controlled conditions, are fuzzy. Researchers have reported good agreement for specific genes identified by microarrays and subsequent testing with northern blot analyses [51] and quantitative PCR [52]. Of course, the relationship between assessed mRNA levels as identified in microarray experiments and the concentrations of the respective proteins is much more complex and less clear [53,54]. More troubling is the reported lack of concordance of microarray data generated from different platforms (oligonucleotides, cDNA arrays) and manufacturers [55–58], although Yuen *et al.* [52] found good concordance between two different oligonucleotide array platforms and with quantitative PCR. These papers point out the need for standardization among microarray providers in their methods of image collection, validation, normalization and analysis. These issues of indistinct results and the lack of concordance suggest, first, that extreme caution should be used in any meta-analysis of gene expression patterns across different studies, and, second, that microarray results should always be validated using more quantitative methods before conclusions should be drawn for any specific gene.

Methods for assessing specific mRNA levels for individual genes have been available for several years. Recent techniques have been based on the use of PCR to amplify specific gene targets from a heterogeneous pool of transcripts. All procedures begin with the use of the enzyme reverse transcriptase to convert the mRNA pool into a hopefully representative pool of cDNAs. The use of RT–PCR has significant advantages over older technologies, not least of which is that mRNA expression can be assayed from minute samples, even single cells [59]. Although quantitative gene expression analysis using RT–PCR is relatively easy to perform and scalable to large numbers of samples, it has one serious limitation in that stochastic errors that are inherent in the methodology are amplified to the same extent as the target gene of interest [60–62]. This is particularly the case in competitive or end-point analysis PCR where the quantity of a given gene transcript is estimated by comparing PCR product yields with carefully developed internal standards. A more recent technique that enables researchers to view PCR amplification with each cycle is faster and easier, and allows for the quantification of the target gene at a point where the synthesis of PCR product is limited only by the amount of starting material rather than by other factors such as decreasing primer concentrations and enzyme stability, as is the case for end-point analysis [63]. Through the use of fluorogenic probes [64] or intercalating DNA dyes, the amplification process can be monitored in real-time, enabling the initial concentration of a given gene target to be estimated based on the amplification profiles. Initial copy numbers of mRNA targets are quantified during real-time PCR analysis based on a comparison with some threshold cycle (C_t), defined as the cycle at which fluorescence is determined to be above background signal and the PCR product is increasing exponentially. The threshold cycle is inversely proportional to the log of the initial copy number (Figure 3).

Compared with other QRT–PCR techniques, real-time quantitative PCR requires shorter development times, less post-PCR processing and is less variable and more reproducible [65]. The easiest and least expensive entry into this technology is via the use of DNA dyes such as SYBR® Green I [66]. This methodology is quick to implement, requiring only the addition of the dye to an already optimized PCR protocol. The major disadvantage of DNA dyes such as SYBR® Green I is that they are nonspecific. Any PCR product (including primer dimers) will result in a signal, thus additional steps must be included in the protocol to ensure that only the PCR product of interest is being synthesized and quantified. This can be done by post-PCR gel analysis of the PCR products or the inclusion of a dissociation (melt) curve analysis. In addition, because there is only a single dye, only one gene can be assayed with each PCR reaction. The use of gene-specific fluorogenic probes corrects both of these shortcomings. Fluorogenic probes can be designed to anneal to only a specific gene target and, by using different fluorescent dyes, a single PCR can be multiplexed to assay for 2–4 distinct gene targets. There is now a variety of fluorogenic probe designs available. Most probes are designed with two dyes, one of which reduces or eliminates (quencher dye) the fluorescent signal of the second

dye (reporter dye) when they are held in close molecular proximity to one another. This can be accomplished by placing the two dyes on either end of a single oligonucleotide that then hybridizes to the appropriate gene target within the region being amplified. Fluorescence increases with each amplification cycle as the fluorescent probe is either cleaved into single nucleotides by the DNA polymerase, thus freeing the dyes (TaqMan assay [67–69]), or disrupts the hairpin loop conformation when bound to the PCR product (molecular beacons [70,71]). More recent techniques incorporate the dyes into an additional sequence on one end of one of the PCR primers, which are designed to hybridize to the target as the PCR products are synthesized (scorpion primers [72]). Finally, self-quenching primers can be designed that require only one fluorophore attached to one of the primers (LUX primers [73]). Databases are now available with fluorogenic probe and primer sequences for a variety of genes in humans, mice, rats and other species [74,75].

As is the case for microarrays and other single gene assays, it is useful to normalize the data for differences in mRNA quality, concentrations, RT efficiencies and other experimental errors [62,65,76,77]. The data from real-time QRT–PCR for two samples are by nature relative to one another, although with the development of standards absolute copy numbers for each sample can be estimated. Standard curves can then be constructed using known amounts of the cloned PCR product [78,79].

Applications of QRT–PCR

Uses of real-time quantitative PCR are constantly expanding, but a brief scan of the literature yields studies ranging from assessments of the kinetics of gene expression in response to specific drugs, including prednisolone [80], dexamethasone [81] and ciprofloxacin [82], to examining the role of gene expression for transporters [83–87] and/or metabolizing enzymes [88–91] in drug distribution. Real-time PCR is also rapidly becoming an important means of genotyping for single nucleotide polymorphisms [92–94]. In our laboratory, the working procedure has been to design gene-specific PCR primers and fluorogenic probes for each gene to be assayed. Initially, the real-time PCR is carried out using just the forward and reverse primers and SYBR® Green I dye. The gene-specific fluorogenic probes can be synthesized for those genes confirmed to exhibit differential gene expression. The use of the fluorogenic probes allows for multiplexing as well as eliminating the need for gel analysis of PCR products, because the fluorescent probe reports only the accumulation of specific product. The PCR products are then cloned into a standard plasmid vector. Cloned PCR products are used to establish standard curves of known concentrations for absolute quantitation of gene expression levels.

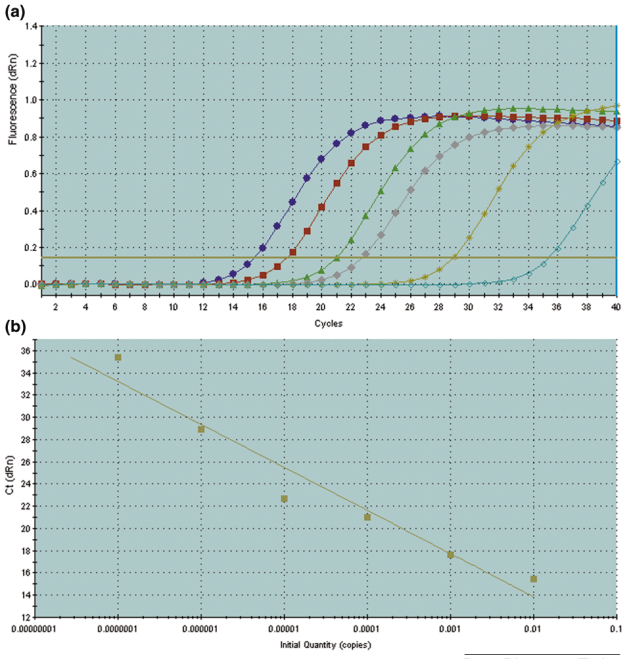

Figure 3. Real-time PCR amplification plot for a series of standards for human cytochrome P450 1A2. Each curve shows increasing fluorescence with each amplification cycle for a dilution series ranging from 10^{-2} to 10^{-7}. The resultant standard curve, which is linear over six orders of magnitude (r^2=0.975), is based on the critical threshold (C_t) values. C_t is determined for each curve as the fractional amplification cycle at which the baseline-corrected normalized fluorescence is determined to be statistically significant above background signal.

Conclusion

In summary, we have combined two genomic technologies as tools in drug discovery and drug development. Initially, microarrays are used to examine large numbers of potential gene targets. Permutation analysis methods are then used to identify those genes that are altered significantly by our experimental treatments. The use of these statistical methods enables us to adjust our list of significant genes based on our concerns with dealing with false positives. Finally, real-time quantitative PCR is then employed to validate our microarray results for specific genes of interest and, because PCR is fast and inexpensive, we can then expand our studies to include more replication and other important parameters such as differing dosage regimens and additional time points.

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