Similarity in Network Structures for in vivo and in vitro Data from the Japanese Toxicogenomics Project

Ryan Gill[1], Somnath Datta[2], Susmita Datta[2]
[1]Department of Mathematics, University of Louisville, Louisville, KY 40292, USA
[2]Department of Bioinformatics and Biostatistics, University of Louisville, Louisville, KY 40202, USA

1. Introduction We provide a partial answer to the important question in Toxicogenomics whether in-vivo microarray expression data based on animal studies can be replaced by in-vitro data. We consider the TGP dataset which contains over 21,000 arrays for rats treated with mainly human drugs and profiled using the Affymetrix RAE230_2.0 GeneChip®. The main target organ profiled is liver. In a previous study, Uehara et al. (2010) identified the genes commonly up-regulated both *in vivo* and *in vitro* after treatment with three different drugs clofibrate, WY-14643 and gemfibrozil. This study was one of the first to create an *in vivo–in vitro* bridge for the validation of a genomic biomarker with those three compounds. In this analysis, we try to provide a comprehensive view of the *in vivo–in vitro* bridging across all the genes (probe sets) for all the 131 drugs provided in the challenge data. Moreover, our approach is not only to observe the similarities in gene expressions of individual genes but to identify the similarities of the network connectivity of all the similar genes across all the chemicals. Methodologically, we consider this question from a statistical perspective and apply a significance test to examine if there is a difference between the genomic networks for the two different types (*in vivo*/*in vitro*) after accounting for different dosages of the drugs, and sacrifice times of the rats. In order to construct the networks of genes and then finding the differences/similarities of the networks for the two types we use the approach similar to the framework for differential network analysis described in our earlier work in Gill et al. (2010). Construction of the networks for each type of data is based on a connectivity score measuring the association between each pair of genes. We apply a connectivity score constructed using a partial least squares (PLS) method that captures the predictability of each gene's expression from a pairing gene after adjusting for other genes and additional covariables (such as dosage) and thus extending our earlier approach to network and differential network analysis (Pihur et al., 2008; Gill et al., 2010; Gill et al., 2012).

In order to study the expression pattern and the network structures, important data preprocessing is required to account for type, dose, and sacrifice time effects. There are substantial differences between the expression values of the MAS5 preprocessed data from the *in vivo* and *in vitro* samples and any naive attempt (such as a gene by gene t-test) might find that all genes are significantly differentially expressed in the two types. We build in the additional preprocessing in our linear model (ANOVA) for log-gene expressions. Similarly, these effects are included in our model for the computation of the PLS scores for the network analysis. These are detailed in the next section.

2. Data We analyze part of the challenge dataset from the Japanese Toxicogenomics Project and compare the MAS5 preprocessed data from the "single dose study *in vivo* experiment using Sparague-Dawley rats" with the "*in vitro* study using hepatocytes from Sparague-Dawley rats" for 131 drugs. The *in vivo* dataset for each drug has microarray expression

values of 31099 genes for 48 rats at four different dose concentrations (control, low, middle, and high) and four different sampling times (3, 6, 9, and 24 hours) with three observations at each combination of the levels for these factors. The in-vitro dataset for each drug has microarray expression values of the same genes for 24 rats at four dose concentrations with the same labels and three different sampling times (2, 8, and 24 hours) with two observations at each combination of the levels. The possibility of using the FARM preprocessed data was also considered, but many of the drugs have many genes with expression value 0 for all observations which precludes the use of regression or even correlation methods since there is no variation in the value of these variables.

3. Methods First, we used a nested ANOVA model to assess the effects of *TYPE* (*in vivo*/*in vitro*), drug dose (*DOSE*) , and sacrifice time (*SAC*) on the expression levels of 31099 genes for each drug. Specifically, for each drug the mean expression value for the ith observation for the gth gene is modeled as

$$\mu_{ig} = TYPE_{ig} + (TYPE{*}SAC{*}DOSE)_{ig}.$$

Before fitting the ANOVA model we take the logarithm of the centered expression levels; the logarithm of the expression values are centered with respect to all genes of the given type. For each drug, the p-values for *TYPE* are computed for each gene under the assumption that the expression values follow a normal distribution with homogeneous error variance. We use these preliminary ANOVA analysis to determine the genes for which the expression are not significantly different for two different types (*in vivo* vs. *in vitro*) at a pairwise type 1 error rate of 0.05. Summarizing the results for all the drugs we find there are 473 genes for which the *TYPE* effect is not significant for at least 80% of the drugs. In other words, the expression profiles of this common set of genes appear to be similar for many of the drugs. Thus, these 473 genes can be taken as common bridging genes between *in vivo* and *in vitro* studies across a great majority of the drugs. However, as the genes do not work independently we want to construct the network of those genes and check their differential behavior across two types.

The tests described in this section are based on connectivity scores s_{ik} which measures the association between the ith and kth genes in a network. Our earlier methods (Gill et al., 2010) for differential network connectivity are modified to allow for additional covariates. We estimate the coefficients for these additional covariates at the same time that the coefficients used to compute the connectivity scores are obtained. Let x_i be the centered and scaled n-dimensional expression vector for the ith gene. The method of computing the PLS scores that is described in Pihur et al. (2008) uses separate PLS models $x_i = \sum_{j \neq i} b_{ij} x_j +$ error, for each gene i. However, in the present context, adjustments for additional effects such as the dose levels are needed; thus we create additional covariate vectors z_1, ..., z_m and fit a set of linear models of the form $x_i = \sum_{k=1}^{k} a_{ik} z_k + \sum_{j \neq i} b_{ij} x_j +$ error. PLS regression is used to estimate the coefficients $a_{i1}, ..., a_{im}, b_{i1}, ..., b_{i,i-1}, b_{i,i+1}, ..., b_{ip}$ based on the design matrix formed by the covariates in the PLS model. The PLS scores are computed based on the estimates $b_{i1}, ..., b_{i,i-1}, b_{i,i+1}, ..., b_{ip}$. The details of the method for computing the PLS regression estimates of the regression coefficients and their conversion to PLS scores are omitted in this extended abstract; these were along the same lines as Pihur et al. (2008). A symmetrized estimate of regression coefficient b_{ij} is taken as the PLS association score $s_{ik} = (\widehat{b}_{ij} + \widehat{b}_{ji})/2$.

Once the connectivity scores are computed for each network, a permutation test is performed to test for differential connectivity of the class of all genes or the test for a single gene. Let $s_{ik}^{(1)}$ and $s_{ik}^{(2)}$ denotes the connectivity scores between genes i and k for networks 1 and 2, respectively. The test statistic for the class of all genes $\mathcal{F}$ with cardinality f is

$$\Delta = \frac{1}{f(f-1)} \sum_{i \neq j \in \mathcal{F}} D(s_{ik}^{(1)}, s_{ik}^{(2)}) \tag{1}$$

and the test statistic for a single gene g is

$$d(g) = \frac{1}{p-1} \sum_{i \neq g} D(s_{ig}^{(1)}, s_{ig}^{(2)}), \tag{2}$$

where D computes the distance between the connectivity scores. We have worked with the L_1-distance $D(s^{(1)}, s^{(2)}) = |s^{(1)} - s^{(2)}|$ rather than the more commonly used L_2-distance leading to a more robust analysis. The permutation test is performed by randomly assigning the labels to each observation in the data set formed by combining the observations from both networks.

4. Results For each of the 131 drugs, tests for differential connectivity of the networks on the set of all 473 non-differentially expressed genes (1) were performed using 1000 permutations based on the L_1 distance function and the PLS connectivity scores. No significant differences in the overall connectivity scores of the networks of this set of 473 genes were found for 77 of the 131 drugs at a 5% significance level. These drugs are listed in Table 1.

acarbose	disopyramide	nimesulide
acetamidofluorene	disulfiram	nitrosodiethylamine
acetaminophen	doxorubicin	papaverine
acetazolamide	enalapril	penicillamine
adapin	erythromycin ethylsuccinate	phenacetin
amitriptyline	ethambutol	phenobarbital
bendazac	ethinylestradiol	phenylanthranilic acid
benziodarone	ethionamide	propylthiouracil
bromoethylamine	etoposide	puromycin aminonucleoside
bucetin	famotidine	quinidine
captopril	fenofibrate	simvastatin
carboplatin	fluphenazine	sulindac
cephalothin	flutamide	sulpiride
chloramphenicol	gentamicin	tamoxifen
chlormadinone	griseofulvin	tannic acid
chlormezanone	hydroxyzine	terbinafine
chlorpheniramine	imipramine	tetracycline
chlorpromazine	labetalol	theophylline
chlorpropamide	lomustine	thioridazine
ciprofloxacin	lornoxicam	ticlopidine
clomipramine	mefenamic acid	tiopronin
colchicine	meloxicam	tolbutamide
cyclosporine A	metformin	triamterene
danazol	methyltestosterone	triazolam
dantrolene	mexiletine	trimethadione
diltiazem	nifedipine	

Table 1: Drugs with similar connectivity scores in the two networks.

Even among the 54 drugs for which the set of all genes are significantly different in terms of overall network connectivities, there are many genes that are not significantly different in terms of individual connectivity scores in the two networks at a 5% level. Tests for the significance difference of the connectivity score of each individual gene within the network (2) were performed for the 54 drugs, and there were 35 genes that were not differentially connected for at least 70% of the drugs. These genes are shown in Table 2.

GENE	prop.	GENE	prop.	GENE	prop.	GENE	prop.	GENE	prop.
1385656_at	0.833	1397371_at	0.759	1395446_at	0.741	1381550_at	0.722	1392859_at	0.704
1395874_at	0.815	1396604_at	0.759	1375063_at	0.741	1370626_at	0.722	1388033_at	0.704
1378788_at	0.796	1392389_at	0.759	1396731_at	0.722	1398741_at	0.704	1385031_at	0.704
1396340_at	0.778	1391493_at	0.759	1385655_at	0.722	1398675_at	0.704	1383272_at	0.704
1393711_at	0.778	1368887_at	0.759	1385589_at	0.722	1397850_at	0.704	1383195_at	0.704
1391313_at	0.778	1368854_at	0.759	1384683_at	0.722	1397720_at	0.704	1381502_at	0.704
1398707_at	0.759	1397339_at	0.741	1384061_at	0.722	1395490_at	0.704	1377391_at	0.704

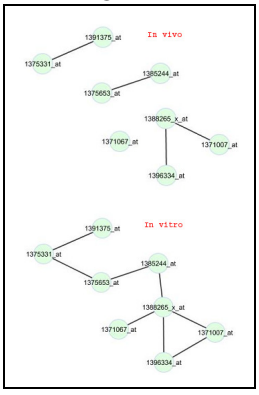
Table 2: Genes not differentially expressed for at least 70% of the remaining 54 drugs. The respective gene names (probe set IDs) and proportion of drugs with similar connectivity scores for that gene in the *in vivo* and *in vitro* networks.

In order to characterize the 473 genes which have shown no significant difference between the *in vivo* and *in vitro* types with more than 80% of the drugs we used functional annotation tool DAVID (Huang et al., 2009a; 2009b). Results of that analysis for the top five functional clusters out of the 473 genes are given in Table 3. Most of the genes in the first functional cluster are involved in neuron development, neuron differentiation, neuron projection morphogenesis and cell morphogenesis activities. The genes in the second most important cluster are involved with proteins in cell-cell junctions of multi-cellular species and also most of them are associated with some synaptic activities. The third most important functional cluster of the genes are associated with epidermal growth factor (EGF) proteins.

Cluster	Enrichment Score	% of drugs
1	4.05	87
2	3.08	92
3	2.20	90
4	1.49	92
5	1.48	95

Table 3: Tests of differential connectivity for the top 5 clusters obtained from the DAVID Functional Annotation Tool. The last column shows the percentages of drugs for which the corresponding sub-networks were not significantly different.

Figure 1: *In vivo* and *in vitro* networks for cluster 4 and the drug phenylbutazone. Edges are displayed for gene pairs with connectivity scores (rescaled so that the largest score for the network is 1 in magnitude) greater than 0.5 in magnitude.



Next, we reconstructed the networks separately for each functional cluster. These networks had fewer significant differences between the *in vivo* and *in vitro* types than the overall

networks. As seen in the Table 3, the difference between the *in vivo* and *in vitro* networks are not statistically significant for at least 87% of the drugs among these top five clusters.

We also annotated 35 genes for each of which the individual network connectivity score between the *in vivo* and *in vitro* types remained unchanged in spite of having significantly different total gene set network connectivity scores under the treatment of 54 drugs. With DAVID annotation tool we figured that all these 35 genes are in one functional cluster and they are associated with cellular macromolecular complex assembly.

Lastly, we wanted to illustrate how these sub-networks behave for a given drug. Figure 1 illustrates the constructed *in vivo* and *in vitro* networks for the genes in cluster 4 for phenylbutazone, a non-steroidal anti-inflammatory drug (NSAID). For these networks, the test for differential connectivity is not significant (p-value is 0.42). All edges in the *in vivo* network also appear in the *in vitro* network, and only 4 edges in the *in vitro* network do not appear in the *in vivo* network.

5. Conclusion A comprehensive view of the *in vivo* - *in vitro* bridge of the genes using the rat microarray TGP study under all the drugs is undertaken. We not only provide the similarity of individual gene expression pattern but also that of the association networks under *in vivo* and *in vitro* experiments. The systems are scrutinized in terms of overall network connectivity and also in terms of individual gene connectivity. We use PLS based association scores adjusted for sacrifice time and dosage followed by a permutation based statistical test with those scores. Since we are trying to identify genes that are not different, a conservative approach in this context will be not to apply a multiple testing p-value correction unlike typical gene expression studies where the goal is to identify genes that are differentially expressed and/or connected under two biological conditions. It is interesting to observe that, similar to Uehara et al. (2010) who studied three of the drugs, none of the bridging genes that we found are involved with cell proliferation and apoptosis.

A potential limitation of our study is that our findings are based on a specific type of statistical model. In the future we plan to undertake additional investigation where networks are constructed by fitting other types of predictive models such as lasso (Tibshirani, 1996) and elastic net (Zou and Hastie, 2005) and the results are compared.

The findings must be interpreted carefully. First of all, we have highlighted the genes which were not significantly different. However it does not quite imply that *in vivo* and *in vitro* studies are completely interchangeable since there are genes that show differential expression and network profiles in the two networks. Furthermore, lack of statistical significance does not necessarily imply that the objects under comparison are indeed equal.

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