

# Gene Set Analysis in R – the GSAR Package

Yasir Rahmatallah<sup>1</sup> and Galina Glazko<sup>2</sup>

Division of Biomedical Informatics,  
University of Arkansas for Medical Sciences,  
Little Rock, AR 72205.

<sup>1</sup>yrahmatallah@uams.edu, <sup>2</sup>gvglazko@uams.edu

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## Contents

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<b>1</b>	<b>Introduction</b>	<b>2</b>
<b>2</b>	<b>Minimum spanning trees</b>	<b>2</b>
2.1	First MST	2
2.2	MST2 for correlation networks	3
<b>3</b>	<b>Multivariate generalizations using MST</b>	<b>3</b>
3.1	Wald-Wolfowitz test	3
3.2	Kolmogorov-Smirnov test	4
<b>4</b>	<b>Gene sets net correlations analysis</b>	<b>4</b>
4.1	Method	4
4.2	The problem of zero standard deviation	5
<b>5</b>	<b>Application to RNA-Seq counts</b>	<b>6</b>
<b>6</b>	<b>Case studies</b>	<b>6</b>
6.1	The p53 dataset	6
6.1.1	Introduction	6
6.1.2	Filtering and normalization	6
6.1.3	GSA	6
6.2	The ALL dataset	9
6.2.1	Introduction	9
6.2.2	Filtering and normalization	9
6.2.3	Selected gene set	10
6.3	The Pickrell dataset	10
6.3.1	Introduction	10
6.3.2	Filtering and normalization	13
6.3.3	Testing selected pathways	13
<b>7</b>	<b>Session info</b>	<b>14</b>

# 1 Introduction

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This vignette gives an overview of the *R* package *GSAR* which provides a set of multivariate statistical tests for self-contained gene set analysis (GSA). *GSAR* consists of two-sample multivariate nonparametric statistical methods testing a null hypothesis against specific alternative hypotheses, such as differences in shift, scale or correlation structure. It also offers a graphical visualization tool for correlation networks to examine the change in the net correlation structure of a gene set between two conditions based on the minimum spanning trees. The package implements the methods proposed in [1, 2, 3] which were thoroughly tested using simulated and microarray datasets in [1] and [2]. These methods (except *RKStest*) can also be applied to RNA-Seq count data given that proper normalization which accounts for both the within-sample differences (gene lengths) and between-samples differences (library sizes) is used. This vignette begins with a brief overview of the theoretical concepts behind the methods, and then gives a number of fully worked case studies, from microarrays to RNA-Seq count data.

Many methodologies for testing the differential expression of gene sets have been suggested and are collectively named gene set analysis (GSA). GSA can be either *competitive* or *self-contained*. Competitive approaches compare a gene set against its complement which contains all genes excluding the genes in the set, and self-contained approaches compare whether a gene set is differentially expressed (DE) between two phenotypes. Competitive GSA approaches are influenced by the genomic coverage and the filtering of the data and can increase their power by the addition of unrelated data and even noise [4]. Due to these problems, package *GSAR* focuses on self-contained methods only. The possibility to formulate different statistical hypotheses by using different test statistics with self-contained approaches enables the formulation and exploration of different biological hypotheses [2]. For GSA, testing hypotheses other than the equality of the mean expression vectors remains underexplored. Package *GSAR* provides a set of methods to test a null hypothesis against specific alternatives, such as differential shift or mean (function *KStest*), scale or variance (function *RKStest*) or correlation structure (function *GSNCAtest*).

Most of the tests available in package *GSAR* (all except *GSNCAtest*) are network or graph-based. *GSAR* handles graphs using the *igraph* class from package *igraph* [5]. *GSAR* also invokes some functions from package *igraph* in its methods implementation and uses the *plot* method for class *igraph* for visualizing the generated graphs.

Data packages *ALL*, *GSVAdata* and *tweeDEseqCountData* which contain datasets are necessary for running the examples and case studies in this document. Package *GSAR* itself contains one preprocessed dataset to illustrate the analyses, which was employed in the article introducing the gene sets net correlations analysis (GSNCA) method [1]. Other packages necessary for running the examples and case studies in this document are packages *MASS*, *GSEABase*, *annotate*, *org.Hs.eg.db*, *genefilter*, *hgu95av2.db* and *edgeR*. The analysis will start by loading package *GSAR*

```
> library(GSAR)
```

In what follows we consider two  $p$ -dimensional vectors of measurements  $X = (X_1 \dots X_{n_1})$  and  $Y = (Y_1 \dots Y_{n_2})$  to be independent and identically distributed with the distribution functions  $F_x$ ,  $F_y$ , mean vectors  $\bar{X}$ ,  $\bar{Y}$  and  $p \times p$  covariance matrices  $S_x$  and  $S_y$ .

## 2 Minimum spanning trees

---

### 2.1 First MST

The pooled multivariate ( $p$ -dimensional) observations  $X$  and  $Y$  can be represented by an edge-weighted graph  $G(V, E)$  where  $V$  is the set of vertices in the graph with each vertex corresponding to one observation and  $E$  is the set of edges connecting pairs of vertices. The complete graph of  $X$  and  $Y$  has  $N = n_1 + n_2$  vertices and  $N(N-1)/2$  edges. The weights of the edges are estimated by the Euclidean distances between pairs of observations in  $R^p$ .

The minimum spanning tree (MST) is defined as the acyclic subset  $T_1 \subseteq E$  that connects all vertices in  $V$  and whose total length  $\sum_{i,j \in T_1} d(v_i, v_j)$  is minimal. Each vertex in the graph corresponds to a  $p$ -dimensional observation from  $X$  or  $Y$ . The MST provides a way of ranking the multivariate observations by giving them ranks according to the positions of their corresponding vertices in the MST. The purpose of this ranking is to obtain the strong relationship between observations differences in ranks and their distances in  $R^p$ . The ranking algorithm can be designed specifically to confine a particular alternative hypothesis more detection power [2]. Three tests in package *GSAR* are based on MST: *WWtest*, *KStest* and *RKStest*.

The following example generates a feature set of 20 features and 40 observations using the random multivariate normal data generator from package [MASS](#), creates a graph object from the data and obtain its MST using functions from package [igraph](#).

```
> library(MASS)
> set.seed(123)
> nf <- 20
> nobs <- 40
> zero_vector <- array(0,c(1,nf))
> cov_mtrx <- diag(nf)
> dataset <- mvrnorm(nobs, zero_vector, cov_mtrx)
> Wmat <- as.matrix(dist(dataset, method="euclidean", diag=TRUE,
+ upper=TRUE, p=2))
> gr <- graph.adjacency(Wmat, weighted=TRUE, mode="undirected")
> mst <- minimum.spanning.tree(gr)
```

## 2.2 MST2 for correlation networks

The second MST is defined as the MST of the reduced graph  $G(V, E - T_1)$ . We denote the union of the first and second MSTs by MST2. Each vertex in the MST2 has a minimum degree of 2 if all the  $p(p-1)/2$  edges between vertices are considered.

The correlation (coexpression) network is defined as the edge-weighted graph  $G(V, E)$  where  $V$  is the set of vertices in the graph with each vertex corresponding to one feature (gene) in the gene set and  $E$  is the set of edges connecting pairs of vertices with weights estimated by the correlation distances. The correlation distances are defined by  $d_{ij} = 1 - |r_{ij}|$  where  $d_{ij}$  and  $r_{ij}$  are respectively the correlation distance and correlation parameter between genes  $i$  and  $j$  [1]. The MST2 of the correlation network gives the minimal set of essential links (interactions) among genes, which we interpret as a network of functional interactions. A gene that is highly correlated with most of the other genes in the gene set tends to occupy a central position and has a relatively high degree in the MST2 because the shortest paths connecting the vertices of the first and second MSTs tend to pass through this gene. In contrast, a gene with low intergene correlations most likely occupies a non-central position in the MST2 and has a degree of 2. This property of the MST2 makes it a valuable graphical visualization tool to examine the full correlation network by highlighting the most highly correlated genes. As an example, the MST2 of the dataset generated in the previous example can be found as follows

```
> ## The input of findMST2 must be a matrix with rows and columns
> ## respectively correspond to genes and columns.
> ## Therefore, dataset must be transposed first.
> dataset <- aperm(dataset, c(2,1))
> MST2 <- findMST2(dataset)
```

## 3 Multivariate generalizations using MST

---

### 3.1 Wald-Wolfowitz test

The Wald-Wolfowitz (WW) tests the null hypothesis  $H_0 : F_x = F_y$  against the alternative  $H_1 : F_x \neq F_y$ . When  $p = 1$ , the univariate WW test begins by sorting the observations from two phenotypes in ascending order and labeling each observation by its phenotype. Then, the number of runs ( $R$ ) is calculated where  $R$  is a consecutive sequence of identical labels. The test statistic is a function of the number of runs and is asymptotically normally distributed.

The multivariate generalization ( $p > 1$ ) suggested in [3] is based on the MST. Similar to the univariate case, in the multivariate generalization of WW test, all edges in the MST connecting two vertices (observations) with different labels are removed and the number of the remaining disjoint trees ( $R$ ) is calculated [3]. The test statistic is the standardized number of subtrees

$$W = \frac{R - E[R]}{\sqrt{\text{var}[R]}}$$

The null distribution of  $W$  is obtained by permuting the observation labels for a large number of times and calculating  $W$  for each time and was found to be asymptotically normal.  $P$ -value is calculated as

$$P - value = \frac{b + 1}{nperm + 1}$$

where  $b$  is the number of permutations giving a more extreme statistic  $W$  than the observed test statistic and  $nperm$  is the total number of permutations. Function `WWtest` performs this test.

### 3.2 Kolmogorov-Smirnov test

When  $p = 1$ , the univariate Kolmogorov-Smirnov (KS) test begins by sorting the observations from two phenotypes in ascending order. Then observations are ranked and the quantity

$$d_i = \frac{r_i}{n_1} - \frac{s_i}{n_2}$$

is calculated where  $r_i$  ( $s_i$ ) is the number of observations in  $X$  ( $Y$ ) ranked lower than  $i$ ,  $1 \leq i \leq N$ . The test statistic is the maximal absolute difference  $D = \max|d_i|$ . The null distribution of  $D$  is obtained by permuting the observation labels for a large number of times and calculating  $D$  for each time.  $P$ -value is calculated in exactly the same way as before for the WW test.

The ranking scheme can be designed to confine a specific alternative hypothesis more power. Two possibilities are available: First, if the null hypothesis  $H_0 : \bar{X} = \bar{Y}$  is tested against the alternative  $H_1 : \bar{X} \neq \bar{Y}$ , the MST is rooted at a node with the largest geodesic distance and the rest of the nodes are ranked according to the *high directed preorder* (HDP) traversal of the tree [3]. Function `KStest` performs this test. Second, if the null hypothesis  $H_0 : var(X) = var(Y)$  is tested against the alternative hypothesis  $H_1 : var(X) \neq var(Y)$ , the MST is rooted at the node of smallest geodesic distance (centroid) and nodes with largest depths from the root are assigned higher ranks. Hence, ranks are increasing *radially* from the root of the MST. Function `RKStest` performs this test.

The MST found in the previous example is shown in Figure 1 where vertices from group 1 are in green and vertices from group 2 are in yellow. Ranking vertices in the graph according to the HDP traversal of the MST can be done using function `HDP.ranking`

```
> HDP.ranking(mst)
```

```
[1] 23 22 10 18 21 19 36 5 33 37 31 27 11 12 32 8 40 28 29 14 4 7 6 3 35
[26] 16 17 15 30 34 20 38 24 25 2 13 9 1 26 39
```

## 4 Gene sets net correlations analysis

### 4.1 Method

Gene sets net correlations analysis (GSNCA) is a two-sample nonparametric multivariate differential coexpression test that accounts for the correlation structure between features (genes). The test assigns weight factors  $w_s$  to genes under one condition and adjust these weights simultaneously such that equality is achieved between each genes's weight and the sum of its weighted correlations with other genes  $r_{ij}$  in a gene set of  $p$  genes

$$w_i = \sum_{j \neq i} w_j r_{ij} \quad 1 \leq i \leq p$$

The problem is solved as an eigenvector problem with a unique solution which is the eigenvector corresponding to the largest eigenvalue of the genes' correlation matrix (see [1] for details).

The test statistic  $w_{GSNCA}$  is given by the first norm between the scaled weight vectors  $w^{(1)}$  and  $w^{(2)}$  (each vector is multiplied by its norm) between two conditions

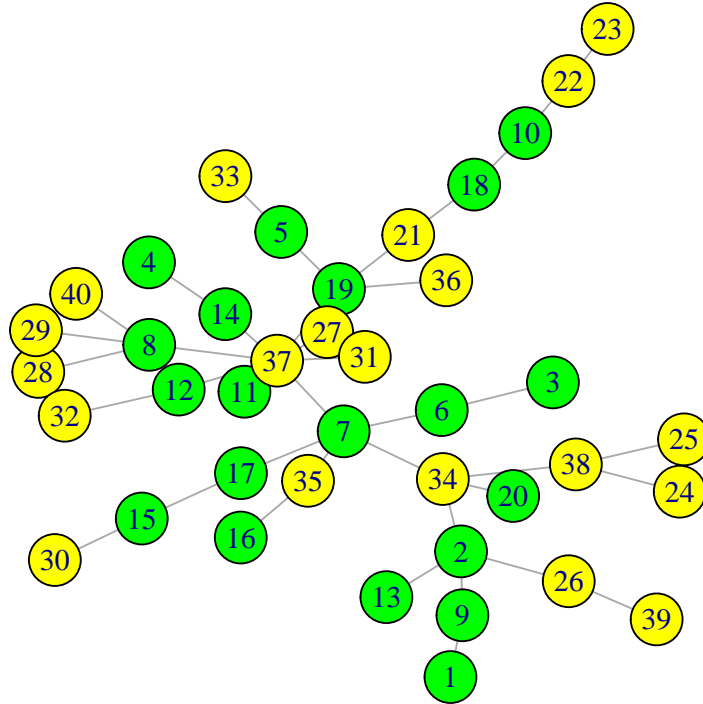


Figure 1: Minimum spanning tree of some random data.

$$w_{GSNCA} = \sum_{i=1}^p \left| w_i^{(1)} - w_i^{(2)} \right|$$

This test statistic tests the null hypothesis  $H_0 : w_{GSNCA} = 0$  against the alternative  $H_1 : w_{GSNCA} \neq 0$ . The performance of this test was thoroughly examined in [1].  $P$ -value is calculated in exactly the same way as before for the WW test.

## 4.2 The problem of zero standard deviation

In rare cases some features in a set may have constant or nearly constant levels across the samples in one or both conditions. Such situation may arise for RNA-Seq counts when some genes with many zero counts are allowed. This results in a zero or a tiny standard deviation. Such case produces an error in command `cor` used to compute the correlations between features. To avoid this situation, standard deviations are checked in advance (default behaviour) and if any is found below a specified minimum limit (default is  $1e-3$ ), the execution stops and an error message is returned indicating the the number of feature causing the problem (if only one the index of that feature is given too). To perform the GSNCA for such set, the features causing the problem must be excluded from the set.

If a feature has nearly a constant level for some samples under both conditions, permuting sample labels may group such samples under one condition by chance and hence produce a standard deviation smaller than the minimum limit. To allow the test to skip such permutations without causing excessive delay, an upper limit for the number of allowed skips can be set (default is 10). If the upper limit is exceeded, an error message is returned.

If the user is certain that the tested feature sets contain no feature with nearly equal levels over many samples (such as the case with filtered microarray data), the checking for tiny standard deviations can be skipped in order to reduce the execution time.

## 5 Application to RNA-Seq counts

---

RNA-Seq data consists of integer counts usually represented by the discrete Poisson or negative Binomial distributions. Therefore, tests designed for microarray data (which follows the continuous normal distribution) can not be applied directly to RNA-Seq data. The nonparametric tests presented in package *GSAR* need no prior distributional assumptions and can be applied to RNA-Seq counts given that proper normalization is used. The normalization should account for the between-samples differences (library size or sequencing depth) and within-sample differences (mainly gene length). The *reads per kilobase per million* (RPKM) is such example. However, due to some limitations, two points must be declared:

- The variance of both the Poisson and negative Binomial distributions, used to model count data, is a function of their mean. Therefore, using the radial KS test (`RKStest`) to detect pathways with differential variance for RNA-Seq counts is not possible.
- RNA-Seq datasets often have many zero counts, therefore, the problem of genes having zero standard deviations in a pathway is frequent and prevent calculating the correlation coefficients necessary to perform the GSNCA. One possible solution is to discard any genes that may have zero or tiny standard deviation and apply GSNCA to the remaining genes in the pathway. Since count data demonstrate much higher differences between genes than microarrays, Spearman's correlation coefficient is preferred (set `cor.method` to "spearman").

## 6 Case studies

---

This Section illustrates the typical procedure for applying the methods available in package *GSAR* to perform GSA. Two microarray and one RNA-Seq datasets are used.

### 6.1 The p53 dataset

#### 6.1.1 Introduction

p53 is a major tumor suppressor protein. The p53 dataset comprises 50 samples of the NCI-60 cell lines differentiated based on the status of the TP53 gene: 17 cell lines carrying wild type (WT) TP53 and 33 cell lines carrying mutated (MUT) TP53 [6, 7]. Transcriptional profiles obtained from microarrays of platform hgu95av2 are available from the Broad Institute's website (<http://www.broadinstitute.org/gsea/datasets.jsp>).

#### 6.1.2 Filtering and normalization

A preprocessed version of p53 dataset is available in package *GSAR* as a *matrix* object. The p53 dataset was downloaded from the Broad Institute's website. Probe level intensities were quantile normalized and transformed to the log scale using  $\log_2(1 + intensity)$ . Probes originally had Affymetrix identifiers which are mapped to unique gene symbol identifiers. Probes without mapping to entrez and gene symbol identifiers were discarded. Probes with duplicate intensities were assessed and the probe with the largest absolute value of t-statistic between WT and MUT conditions was selected as the gene match. Finally, genes were assigned gene symbol identifiers and columns were assigned names indicating whether they belong to WT or MUT group. The columns were sorted such that the first 17 columns are WT samples and the next 33 columns are the MUT samples. This processed version of the p53 dataset was used in the analysis presented in [1].

#### 6.1.3 GSA

GSA are performed on selected C2 curated gene sets (pathways) of the *molecular signatures database* (MSigDB) 3.0 [8]. This list of gene sets is available in package *GSVAdat*. We start by loading the required data

```
> library(GSVAdat)
> data(p53DataSet)
> data(c2BroadSets)
```

c2BroadSets is an object of class *GeneSetCollection* supported by package [GSEABase](#). The genes in the c2BroadSets object have entrez identifiers. Package [org.Hs.eg.db](#) is used to convert the entrez identifiers to gene symbol identifiers. Genes without unique mapping to gene symbol identifiers or that do not exist in the p53 dataset are discarded from the C2 pathways. Finally, we keep only pathways with  $10 \leq p \leq 500$  where  $p$  is the number of genes remaining in the pathways after filtering steps.

```
> library(org.Hs.eg.db)
> library(GSEABase)
> C2 <- as.list(geneIds(c2BroadSets))
> len <- length(C2)
> genes.entrez <- unique(unlist(C2))
> genes.symbol <- array("", c(length(genes.entrez), 1))
> x <- org.Hs.egSYMBOL
> mapped_genes <- mappedkeys(x)
> xx <- as.list(x[mapped_genes])
> for (ind in 1:length(genes.entrez)){
+   if (length(xx[[genes.entrez[ind]]])!=0)
+     genes.symbol[ind] <- xx[[genes.entrez[ind]]]
+ }
> ## discard genes with no mapping to gene symbol identifiers
> genes.no.mapping <- which(genes.symbol == "")
> if(length(genes.no.mapping) > 0){
+   genes.entrez <- genes.entrez[-genes.no.mapping]
+   genes.symbol <- genes.symbol[-genes.no.mapping]
+ }
> names(genes.symbol) <- genes.entrez
> ## discard genes in C2 pathways which do not exist in p53 dataset
> p53genes <- rownames(p53DataSet)
> remained <- array(0, c(1, len))
> for (k in seq(1, len, by=1)) {
+   remained[k] <- sum((genes.symbol[C2[[k]]] %in% p53genes) &
+   (C2[[k]] %in% genes.entrez))
+ }
> C2 <- C2[(remained>=10)&&(remained<=500)]
> pathway.names <- names(C2)
> c2.pathways <- list()
> for (k in seq(1, length(C2), by=1)) {
+   selected.genes <- which(p53genes %in% genes.symbol[C2[[k]]])
+   c2.pathways[[length(c2.pathways)+1]] <- p53genes[selected.genes]
+ }
> names(c2.pathways) <- pathway.names
> path.index <- which(names(c2.pathways) == "LU_TUMOR_VASCULATURE_UP")
```

c2.pathways is now a list with each entry being a named list of the genes (gene symbol identifiers) forming one C2 pathway. To demonstrate the use of different tests, we consider the interesting C2 pathway LU TUMOR VASCULATURE UP used in [1] to demonstrate the GSNCA.

```
> target.pathway <- p53DataSet[c2.pathways[["LU_TUMOR_VASCULATURE_UP"]],]
> group.label <- c(rep(1,17), rep(2,33))
> WWresult <- WWtest(target.pathway, group.label)
> KSresult <- KStest(target.pathway, group.label)
> RKSresult <- RKStest(target.pathway, group.label)
> GSNCAresult <- GSNCAtest(target.pathway, group.label)
> WWresult$p.value
[1] 0.0689
> KSresult$p.value
[1] 0.049
> RKSresult$p.value
```

```
[1] 0.173
```

```
> GSNCAresult$p.value
```

```
[1] 0.029
```

The questions addressed by these tests were the identification of gene sets expressed with different distributions, means, variances or correlation structure between two conditions. At a significance level 0.05, the targeted pathway shows a statistical evidence of being differentially coexpressed only. The MST2s of the correlation network for WT and MUT groups are shown in Figure 2, generated by function `plotMST2.pathway`

```
> plotMST2.pathway(p53DataSet[c2.pathways[[path.index]],],
+ group=c(rep(1,17), rep(2,33)), name="LU_TUMOR_VASCULATURE_UP",
+ legend.size=0.9, label.size=1.2, cor.method="pearson")
```

### Pathway: LU\_TUMOR\_VASCULATURE\_UP

There are 22 genes in this pathway

**Group 1**  
**Hub Gene (group 1): TNFAIP6**  
**Weight Factor: 1.362**  
**Hub Gene (group 2): VCAN**  
**Weight Factor: 1.165**

**Group 2**  
**Hub Gene (group 2): VCAN**  
**Weight Factor: 1.465**  
**Hub Gene (group 1): TNFAIP6**  
**Weight Factor: 0.979**

**MST2 for group 1**

**MST2 for group 2**

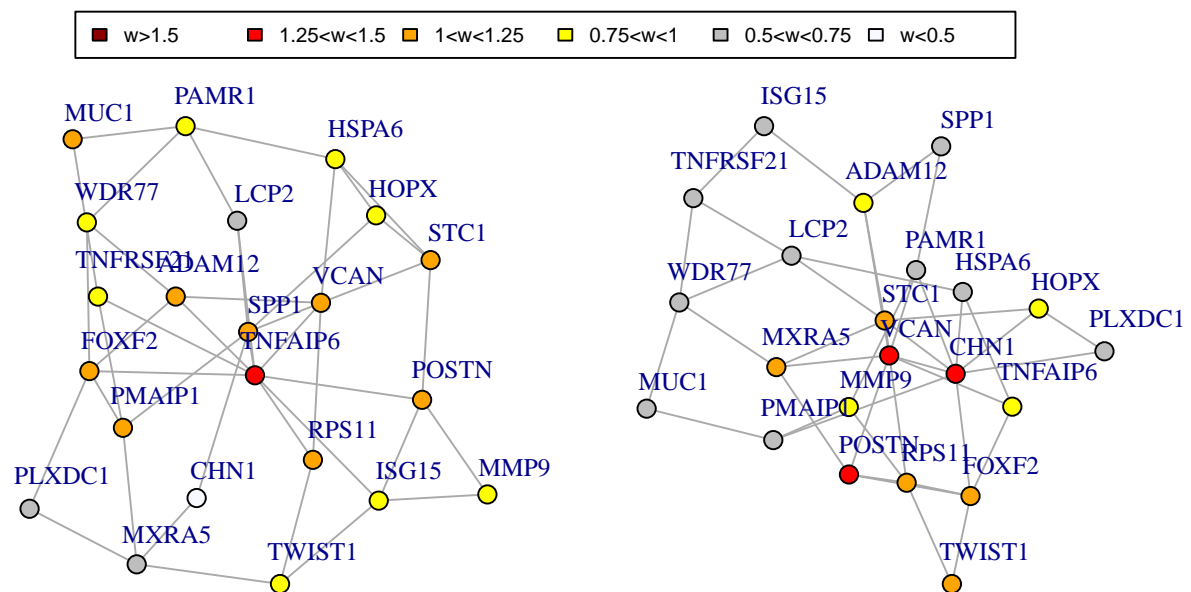


Figure 2: MST2s of LU TUMOR VASCULATURE UP correlation network, (left) WT (right) MUT.

The targeted pathway comprises genes over-expressed in ovarian cancer endothelium [9]. Gene TNFAIP6 (tumor necrosis factor,  $\alpha$ -induced protein 6) identified by GSNCA as a hub gene for WT group and visualized using MST2



(Figure 2, left panel) was found 29.1-fold over-expressed in tumor endothelium in the original study and was suggested to be specific for ovarian cancer vasculature. This indicates that gene TNFAIP6 can be an important regulator of ovarian cancer, and identifying it as a hub by GSNCA enhances the original observation. When p53 is mutated (Figure 2, right panel) the hub gene is VCAN, containing p53 binding site and its expression is highly correlated with p53 dosage [10]. Therefore, both hub genes provide adequate information about the underlying biological processes.

If testing all the gene sets in the `c2.pathways` list is desired, a loop can be constructed to extract one pathway at a time and perform the desired tests similar to what has been shown above.

## 6.2 The ALL dataset

### 6.2.1 Introduction

This dataset consists of microarrays (platform `hgu95av2`) from 128 different individuals with acute lymphoblastic leukemia (ALL). There are 95 samples with B-cell ALL [11] and 33 with T-cell ALL [12]. We consider B-cell type only and compare tumors carrying the BCR/ABL mutations (37 samples) to those with no cytogenetic abnormalities (42 samples). The Bioconductor package [ALL](#) provides the ALL dataset with samples normalized using the *robust multiarray analysis* (RMA) procedure [13].

### 6.2.2 Filtering and normalization

Affymetrix probe identifiers were mapped to unique gene symbol identifiers. Probes without mapping to entrez and gene symbol identifiers were discarded. Probes with duplicate intensities were assessed and the probe with the largest absolute value of t-statistic between normal (NEG) and mutation (MUT) conditions was selected as the gene match. Finally, genes were assigned gene symbol identifiers.

```
> library(Biobase)
> library(genefilter)
> library(annotate)
> library(hgu95av2.db)
> library(ALL)
> data(ALL)
> bcell = grep("^B", as.character(ALL$BT))
> types = c("NEG", "BCR/ABL")
> moltyp = which(as.character(ALL$mol.biol) %in% types)
> ALL_bcrneg = ALL[, intersect(bcell, moltyp)]
> ALL_bcrneg$mol.biol = factor(ALL_bcrneg$mol.biol)
> ALL_bcrneg$BT = factor(ALL_bcrneg$BT)
> nBCR <- sum(ALL_bcrneg$mol.biol == "BCR/ABL")
> nNEG <- sum(ALL_bcrneg$mol.biol == "NEG")
> BCRsamples <- which(ALL_bcrneg$mol.biol == "BCR/ABL")
> NEGsamples <- which(ALL_bcrneg$mol.biol == "NEG")
> ALL_bcrneg <- ALL_bcrneg[,c(BCRsamples, NEGsamples)]
> platform <- annotation(ALL_bcrneg)
> annType <- c("db", "env")
> entrezMap <- getAnnMap("ENTREZID", annotation(ALL_bcrneg),
+ type=annType, load=TRUE)
> symbolMap <- getAnnMap("SYMBOL", annotation(ALL_bcrneg),
+ type=annType, load=TRUE)
> filtered <- nsFilter(ALL_bcrneg, require.entrez=TRUE,
+ remove.dupEntrez=FALSE, require.symbol=TRUE, require.GOBP=FALSE,
+ var.func=IQR, var.filter=FALSE, var.cutoff=0.5)
> filtered.set <- filtered$set
> probe.names <- featureNames(filtered.set)
> rr <- rowttests(filtered.set, as.factor(ALL_bcrneg$mol.biol), tstatOnly=TRUE)
> fL <- findLargest(probe.names, abs(rr$statistic), platform)
```

```

> filtset2 <- filtered.set[fL,]
> affymetrix.probe.names <- featureNames(filtset2)
> gene.symbols <- lookUp(affymetrix.probe.names, platform, "SYMBOL")
> featureNames(filtset2) <- gene.symbols
> ALLdataset <- exprs(filtset2)

```

### 6.2.3 Selected gene set

Lets examine the C2 pathway KEGG CHRONIC MYELOID LEUKEMIA, knowm to be specifically associated with the BCR/ABL mutation. This pathway has many BCR/ABL-related genes and hence expected to show difference between NEG and MUT conditions.

```

> KCMLpathway <- ALLdataset[c2.pathways[["KEGG_CHRONIC_MYELOID_LEUKEMIA"]],]
> group.label <- c(rep(1,37), rep(2,42))
> WWresult <- WWtest(KCMLpathway, group.label)
> KSresult <- KStest(KCMLpathway, group.label)
> RKSresult <- RKStest(KCMLpathway, group.label)
> GSNCAresult <- GSNCAtest(KCMLpathway, group.label)
> WWresult$p.value
[1] 0.000999
> KSresult$p.value
[1] 0.723
> RKSresult$p.value
[1] 0.424
> GSNCAresult$p.value
[1] 0.005

```

At a significance level 0.05,  $p$ -values show statistical evidences that the pathway is differentially coexpressed and has different distributions between BCR/ABL and NEG conditions. The MST2s of the correlation network for BCR/ABL and NEG groups are shown in Figure 3, generated by function `plotMST2.pathway`

```

> plotMST2.pathway(KCMLpathway, group.label, name="KEGG_CHRONIC_MYELOID_LEUKEMIA",
+ legend.size=0.9, label.size=1, cor.method="pearson")

```

## 6.3 The Pickrell dataset

### 6.3.1 Introduction

The Pickrell dataset of sequenced cDNA libraries generated from 69 lymphoblastoid cell lines derived from unrelated Yoruban Nigerian individuals (YRI) is part of the HapMap project. The original experimental data was published by [14]. Package *tweeDEseqCountData* provides the table of counts for this dataset in the expression set object `pickrell.eset`. This table of counts corresponds to the one in the ReCount repository available at <http://bowtie-bio.sourceforge.net/recount>. Details on the pre-processing steps to obtain this table of counts from the raw reads are provided by [15].

Package *tweeDEseqCountData* provides annotation data for the human genes forming the table in `pickrell.eset` as a data frame object `annotEnsembl63`. *tweeDEseqCountData* also provides two lists of genes (gene sets) with documented sex-specific expression and occurring within the set of genes that form the table of counts in `pickrell.eset`. The first is a set of genes that are located on the male-specific region of chromosome Y, and therefore are over-expressed in males (`msYgenes`). The second is a set of genes, that are escaping X-chromosome inactivation, and therefore are overexpressed in females (`XiEgenes`). These two sets are useful in serving as true positives when GSA are conducted between males and females to detect gene sets that are differentially expressed.

```

> library(tweeDEseqCountData)
> data(pickrell)
> data(annotEnsembl63)

```

Pathway: KEGG\_CHRONIC\_MYELOID\_LEUKEMIA

There are 69 genes in this pathway

**Group 1**  
**Hub Gene (group 1): ARAF**  
**Weight Factor: 1.441**  
**Hub Gene (group 2): BCL2L1**  
**Weight Factor: 1.402**

**Group 2**  
**Hub Gene (group 2): BCL2L1**  
**Weight Factor: 1.463**  
**Hub Gene (group 1): ARAF**  
**Weight Factor: 1.194**

MST2 for group 1

MST2 for group 2

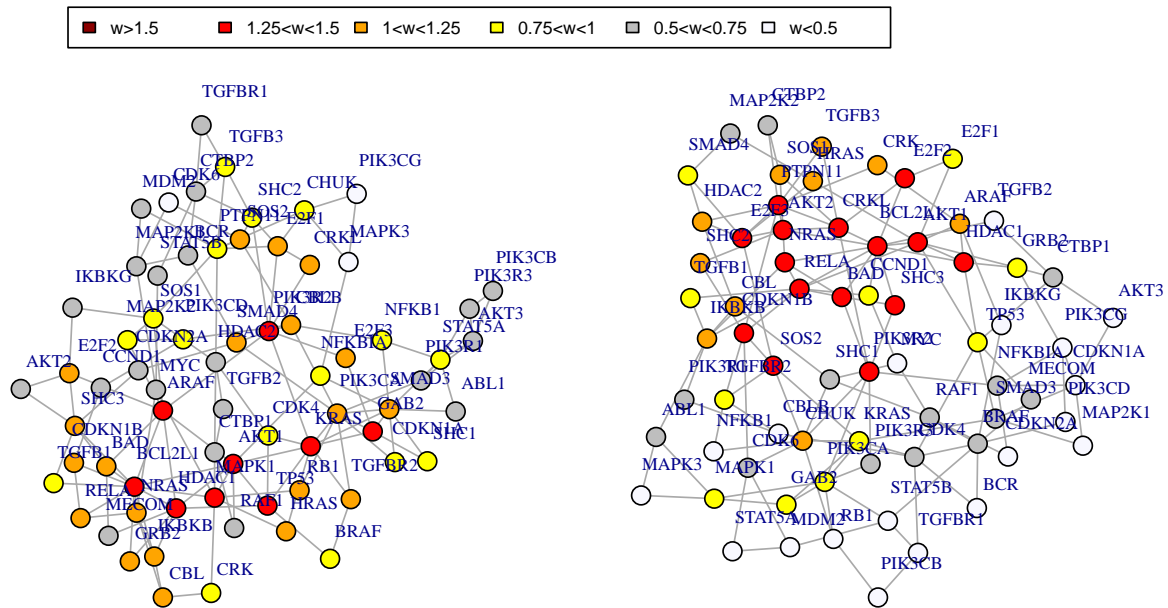


Figure 3: MST2s of pathway KEGG CHRONIC MYELOID LEUKEMIA correlation network, (left) BCR/ABL (right) NEG.

```
> data(genderGenes)
> gender <- pickrell.eset$gender
> pickrell.eset

ExpressionSet (storageMode: lockedEnvironment)
assayData: 52580 features, 69 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: NA18486 NA18498 ... NA19257 (69 total)
  varLabels: num.tech.reps population study gender
  varMetadata: labelDescription
featureData
  featureNames: ENSG00000000003 ENSG00000000005 ... LRG_99 (52580)
```

```

total)
fvarLabels: gene
fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation:

> sampleNames(pickrell.eset)[gender == "male"]

[1] "NA18486" "NA18498" "NA18501" "NA18504" "NA18507" "NA18510" "NA18516"
[8] "NA18519" "NA18522" "NA18853" "NA18856" "NA18862" "NA18871" "NA18913"
[15] "NA19098" "NA19101" "NA19119" "NA19128" "NA19130" "NA19138" "NA19144"
[22] "NA19153" "NA19160" "NA19171" "NA19192" "NA19200" "NA19203" "NA19210"
[29] "NA19239"

> sampleNames(pickrell.eset)[gender == "female"]

[1] "NA18499" "NA18502" "NA18505" "NA18508" "NA18511" "NA18517" "NA18520"
[8] "NA18523" "NA18852" "NA18855" "NA18858" "NA18861" "NA18870" "NA18909"
[15] "NA18912" "NA18916" "NA19093" "NA19099" "NA19102" "NA19108" "NA19114"
[22] "NA19116" "NA19127" "NA19131" "NA19137" "NA19140" "NA19143" "NA19147"
[29] "NA19152" "NA19159" "NA19172" "NA19190" "NA19193" "NA19201" "NA19204"
[36] "NA19209" "NA19222" "NA19225" "NA19238" "NA19257"

> head(annotEnsembl63)

      Symbol Chr      Start      End EntrezID
ENSG00000252775    U7    5 133913821 133913880    <NA>
ENSG00000207459    U6    5 133970529 133970635    <NA>
ENSG00000252899    U7    5 133997420 133997479    <NA>
ENSG00000201298    U6    5 134036862 134036968    <NA>
ENSG00000222266    U6    5 134051173 134051272    <NA>
ENSG00000222924    U6    5 137405044 137405147    <NA>

      Description Length
ENSG00000252775 U7 small nuclear RNA [Source:RFAM;Acc:RF00066]    NA
ENSG00000207459 U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]    NA
ENSG00000252899 U7 small nuclear RNA [Source:RFAM;Acc:RF00066]    NA
ENSG00000201298 U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]    NA
ENSG00000222266 U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]    NA
ENSG00000222924 U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]    NA

      GCcontent
ENSG00000252775    NA
ENSG00000207459    NA
ENSG00000252899    NA
ENSG00000201298    NA
ENSG00000222266    NA
ENSG00000222924    NA

> length(msYgenes)

[1] 32

> length(XiGenes)

[1] 63

We will also extract the set of all X-linked genes that are not escaping inactivation (Xigenes) to use it as a true negative set (not differentially expressed)

> allXgenes <- rownames(annotEnsembl63)[annotEnsembl63$Chr == "X"]
> Xigenes <- allXgenes[!(allXgenes %in% XiGenes)]
> length(Xigenes)

[1] 2249

```

### 6.3.2 Filtering and normalization

Any transcript without entrez identifier or gene length information is discarded. To consider only expressed genes in the analysis, genes with an average *count per million* (cpm) less than 0.1 are discarded.

```
> library(edgeR)
> gene.indices <- which(!(is.na(annotEnsembl63$EntrezID) |
+ is.na(annotEnsembl63$Length)))
> PickrellDataSet <- exprs(pickrell.eset)
> PickrellDataSet <- PickrellDataSet[gene.indices,]
> genes.length <- annotEnsembl63$Length[gene.indices]
> cpm.matrix <- cpm(PickrellDataSet)
> cpm.means <- rowMeans(cpm.matrix)
> cpm.filter <- which(cpm.means > 0.1)
> PickrellDataSet <- PickrellDataSet[cpm.filter,]
> genes.length <- genes.length[cpm.filter]
> rpkm.set <- rpkm(PickrellDataSet, genes.length)
> rpkm.set <- log2(1 + rpkm.set)
```

### 6.3.3 Testing selected pathways

Any gene in msYgenes, XiEgenes, or Xigenes but not found in the filtered dataset is discarded. Then, the remaining gender-related genes in msYgenes and XiEgenes are combined into one gene set (XYgenes).

```
> gene.space <- rownames(rpkm.set)
> msYgenes <- msYgenes[msYgenes %in% gene.space]
> XiEgenes <- XiEgenes[XiEgenes %in% gene.space]
> Xigenes <- Xigenes[Xigenes %in% gene.space]
> XYgenes <- c(msYgenes, XiEgenes)
> length(XYgenes)

[1] 14

> length(Xigenes)

[1] 147
```

The gender-related gene set XYgenes was found differentially expressed with high significance

```
> XYpathway <- rpkm.set[XYgenes,]
> group.label.pickrell <- (gender == "male") + 1
> WWresult <- Wttest(XYpathway, group.label.pickrell)
> KSresult <- KStest(XYpathway, group.label.pickrell)
> WWresult$p.value

[1] 0.000999

> KSresult$p.value

[1] 0.000999
```

while gene set Xigenes showed no such evidence as expected

```
> Xipathway <- rpkm.set[Xigenes,]
> WWresult <- Wttest(Xipathway, group.label.pickrell)
> KSresult <- KStest(Xipathway, group.label.pickrell)
> WWresult$p.value

[1] 0.509

> KSresult$p.value

[1] 0.524
```

To apply the GSNCA, genes with tiny standard deviations must be filtered out first

```
> nrow(XYpathway)
[1] 14

> nrow(Xipathway)
[1] 147

> tiny.sd.XY.female <- which(apply(XYpathway[, group.label.pickrell == 1], 1, "sd") < 1e-3)
> tiny.sd.XY.male <- which(apply(XYpathway[, group.label.pickrell == 2], 1, "sd") < 1e-3)
> tiny.sd.Xi.female <- which(apply(Xipathway[, group.label.pickrell == 1], 1, "sd") < 1e-3)
> tiny.sd.Xi.male <- which(apply(Xipathway[, group.label.pickrell == 2], 1, "sd") < 1e-3)
> length(tiny.sd.XY.female)
[1] 2

> length(tiny.sd.XY.male)
[1] 0

> length(tiny.sd.Xi.female)
[1] 0

> length(tiny.sd.Xi.male)
[1] 0

> apply(XYpathway[, group.label.pickrell == 1], 1, "sd")
ENSG00000183878 ENSG00000154620 ENSG00000198692 ENSG00000157828 ENSG00000173674
      0.000      0.214      0.000      0.206      0.572
ENSG00000186310 ENSG00000169100 ENSG00000189037 ENSG00000215301 ENSG00000147003
      0.162      0.342      0.163      0.773      0.465
ENSG00000182287 ENSG00000169895 ENSG00000196459 ENSG00000169906
      0.502      0.446      0.322      0.143

> if(length(tiny.sd.XY.male) > 0) XYpathway <- XYpathway[-tiny.sd.XY.male,]
> if(length(tiny.sd.XY.female) > 0) XYpathway <- XYpathway[-tiny.sd.XY.female,]
> if(length(tiny.sd.Xi.male) > 0) Xipathway <- Xipathway[-tiny.sd.Xi.male,]
> if(length(tiny.sd.Xi.female) > 0) Xipathway <- Xipathway[-tiny.sd.Xi.female,]
> nrow(XYpathway)
[1] 12

> nrow(Xipathway)
[1] 147
```

Note that two genes (ENSG00000183878 and ENSG00000198692) in XYpathway had zero standard deviations for female samples and were filtered out from XYpathway. These two genes are Y-linked genes and expected to have many zero counts for female samples. Although this filtering step increases the chances of success in performing GSNCA, the existence of many zero counts dispersed over many samples for one or more genes many still cause a problem when a sample permutation groups many zero counts under one condition. The parameter `max.skip` in function `GSNCAtest` allows some tolerance by assigning the maximum number of skipped permutations allowed to avoid the few ones causing the problem. This solution may work or fail depending on the proportion of zero counts in the data. For example, assigning `max.skip` to 100 or more solved the problem for XYpathway, but it did not for Xipathway.

## 7 Session info

---

```
> sessionInfo()
```

```

R version 3.1.1 Patched (2014-09-25 r66681)
Platform: x86_64-apple-darwin10.8.0 (64-bit)

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

attached base packages:
[1] stats4      parallel  stats      graphics  grDevices  utils      datasets
[8] methods     base

other attached packages:
[1] edgeR_3.8.0           limma_3.22.0          tweedEseqCountData_1.3.0
[4] ALL_1.7.0             hgu95av2.db_3.0.0     genefilter_1.48.0
[7] GSVAdat_1.1.0         hgu95a.db_3.0.0       org.Hs.eg.db_3.0.0
[10] RSQLite_0.11.4        DBI_0.3.1             GSEABase_1.28.0
[13] graph_1.44.0          annotate_1.44.0        XML_3.98-1.1
[16] AnnotationDbi_1.28.0  GenomeInfoDb_1.2.0    IRanges_2.0.0
[19] S4Vectors_0.4.0       Biobase_2.26.0        BiocGenerics_0.12.0
[22] MASS_7.3-35          GSAR_1.0.0            igraph_0.7.1

loaded via a namespace (and not attached):
[1] splines_3.1.1  survival_2.37-7  tools_3.1.1      xtable_1.7-4

```

## References

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