Large-scale gene network analysis reveals the significance of extracellular matrix pathway and homeobox genes in acute myeloid leukemia: an introduction to the Pigengene package and its applications

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Supplementary Note 1: The advantages of network analysis. Biological processes in a cell often require intricate coordination between multiple genes and proteins, not just one gene or a single protein. Therefore, the traditional approaches that study associations between individual genes and specific diseases are unable to provide a full understanding of complex biological phenomena.

Although statistical tests such as ANOVA, t-test, and rank products are successfully used in many studies to identify differentially expressed genes, they have a limited statistical power. These approaches fail to pinpoint the biological mechanisms of complicated conditions such as cancer because complex diseases are usually caused by collaboration of more than a few genes. In other words, subtle but coordinated and consistent changes in the expression of a set of functionally related genes can be more important and informative than dramatic changes in the expression of a few individual genes.

Gene network analysis models the interactions between genes in a comprehensive structure. The approaches taken to infer gene or protein networks include ordinary differential equations (ODEs), Boolean networks, cofunction networks, coexpression analysis, and Bayesian networks. Our methodology is inspired by, and builds upon, Bayesian network and coexpression network analyses that have been successfully used for interpreting many biological experiments. While coexpression analysis can potentially model the entire genome, unfortunately, its application is limited due to low accuracy, a deficiency that is rooted in imperfect clustering. Bayesian networks can accurately model complicated probabilistic dependencies between a handful of genes. However, it is very challenging to fit them to the data when the number of genes exceeds hundreds or thousands. We addressed this challenge by comparing expressions at the module level. Our analysis is robust to biological and technical noise because an eigengene is a weighted average expression of several genes, and thus, not affected by random fluctuation in expressions of a few genes.

Supplementary Note 2: Identifying gene modules. The MILE dataset includes expression data of 202 AML and 164 MDS cases measured using Affymetrix microarrays with 54,000 probes (see Methods). Using GEO2R web application, we obtained an R script that ranked all of the 54,000 probes based on their p-values. The script used limma (version 3.22.7) to test for each probe the null hypothesis that it was similarly expressed in AML and MDS (Supplementary Script 1). Consistent with the approach taken by other scholars, we kept all of the top one-third (n=18,200) of the most variable probes in our analysis.

We used Custom CDF (version 15) to map probes to Entrez-gene IDs. The mapping was not one-to-one and we took the following approach to project the data from the probe level to the gene level. First, we excluded the probes that were mapped to multiple Entrez-gene IDs. Out of 18,200 probes, 13,294 remained. Next, among all probes that were mapped to a specific Entrez-gene, the one with the lowest p-value was chosen as the “representative” of that gene. That is, we considered the most differentially expressed probe as the representative of a gene. For genes with only one corresponding probe, this probe was taken as the representative. Our approach resulted in an expression profile with 9,166 probes, each representing to a unique Entrez-gene.

http://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE15061
with expression values for 202 AML and 164 MDS cases. We stored these data in two 9,166 × 202 and 9,166 × 164 matrices.

To identify the gene modules, we applied Weighted correlation network analysis (WGCNA, version 1.41) on the 9,166 × 202 AML expression matrix\(^{35}\) (Supplementary Table 1). Specifically, we used the following parameters to call blockwiseModules() function from WGCNA package. The power (β) parameter was adjusted based on the recommendation of authors using pickSoftThreshold() function with the default value of RsquaredCut=0.85. For better results, we set the parameter maxBlockSize=9166 so that the process was done in only one block. We set TOMType= "unsigned", and used the default values for the rest of the arguments of blockwiseModules(). WGCNA could not confidently assign 4,125 genes to any of the modules because they hardly correlated with any other gene. They were designated as module 0, and excluded from the rest of the analysis.
Supplementary Information

Supplementary Figure 1. The distribution of module sizes. The 33 modules are sorted on the x-axes based on their sizes. The largest and smallest modules consist of 888 and 21 genes, respectively. Module sizes had a mean, median, and standard deviation of 153, 75, and 188, respectively. The plot does not include module 0, the set of 4,125 outlier genes that WGCNA could not confidently assigned to any module.
Supplementary Figure 2. Overrepresentation analysis on modules (part 1). Module 6 is associated with the cell cycle (a), and module 12 with the extracellular region (b). The dashed blue line indicates an adjusted p-value of 0.05. The plots are produced using InnateDB website.
Supplementary Figure 3. Overrepresentation analysis on modules (part 2). Module 14 is associated with cytotoxic pathway (a), Module 15 with DNA replication (b), and module 21 with translation (c).
Supplementary Figure 4. Miller scores. The 33 modules identified using the MILE dataset are sorted on the x-axes based on their sizes. The y-axis shows the percentage of genes in each module that were reported to be related to AML in at least 2 (red), 3 (green), 4 (blue), and 5 (purple) studies according to Miller et al. survey. The HOXA&B module (28) had the highest enrichment as expected because many of HOXA and HOXB genes were reported by scholars to be associated to AML. Supplementary Table 3 includes the numbers corresponding to this plot.
Supplementary Figure 5. Expression of all eigengenes in the MILE dataset. Eigengenes show significantly different pattern in the samples (rows) for the two disease types in the MILE data set. The green strip at the top represents the logarithm of adjusted p-values in base 10. Columns (modules) are clustered based on the similarity of expression in the MILE dataset. The expressions of these eigengenes in the MILE and BCCA datasets are reported in Supplementary Table 4.
Supplementary Note 3: Fitting a Bayesian network to the eigengenes.

A Bayesian network is a statistical model that represents a set of random variables using a directed acyclic graph. Nodes of the network correspond to random variables and the edges (arcs) model their conditional dependencies. An important property of Bayesian networks is that each node conditioned on its parent variables is independent of its non-descendants. In particular, if two nodes are not connected by a directed path, they are conditionally independent.

We trained a Bayesian network to model the probabilistic dependencies between the modules. Each module eigengene was represented by a node (observed random variable). To model the hematological malignancy, we added “Disease” as an observed random variable to the network. For instance in our study, it was equal to 1 for AML, and 0 for MDS. No eigengene was allowed to be a parent of Disease node.

We used bnlearn package to infer the edges and fit the above Bayesian network to the eigengenes. Specifically, we discretized the values of eigengenes into three levels using Hartemink’s method. We used the `bn.boot()` function from the bnlearn package to fit 1000 networks to the discretized data. This function used hill climbing strategy to optimize Bayesian Dirichlet equivalent (BDe) score. Consistent with the approach taken by other scholars, we averaged one-third of the networks with the highest scores to obtain the consensus network. To facilitate applying the above procedure in other studies, we provided `learn.bn()` function in our Pigengene R package (version 0.99.19). In particular, Code 1 reproduces the results presented in this paper. See the package manual for more detail.

**Code 1. Reproducing the Bayesian network**

```r
library(Pigengene) ## version 0.99.19
data(eigengenes33)
amlE <- eigengenes33$aml
mdsE <- eigengenes33$mds
eigengenes <- rbind(amlE, mdsE)
Labels <- c(rep("AML",nrow(amlE)), rep("MDS",nrow(mdsE)))
names(Labels) <- rownames(eigengenes)
learnt <- learn.bn(Data=eigengenes, Labels=Labels,
                   bnPath="bn", bnNum=1000, seed=1, verbose =4)
## Visualize:
d1 <- draw.bn(BN=learnt$consensus1$BN, nodeFontSize=18)
```

The computation does not need more than 2 MB of memory and it is done in one to two days depending on the computer speed. Our package is capable of learning the networks in parallel using a computer cluster. Parallelization results in decreasing the wall-time substantially, dividing it by the number of available compute nodes.
Supplementary Figure 6. Comparing the expression of all genes in the extracellular matrix module. Expressions of every member of module 12, the module associated with the extracellular matrix, are shown in a column. Similar to the extracellular region subset (Fig. 5), these genes are mostly underexpressed in AML compared to MDS. Their variable expressions in some AML cases indicate the heterogeneity of the disease. They have positive weights in the corresponding eigengene except DUSP10 (weight = -0.8).
Supplementary Figure 7. The expression of genes in HOXA&B module in the BCCA dataset. The pattern of gene expressions is similar to the MILE dataset, i.e., the majority of these HOXA and HOXB genes are not expressed in MDS. For clarity, the columns are scaled, and have the same ordering as in Fig. 6.
Supplementary Figure 8. Overrepresented cellular component categories in the extracellular matrix module. The extracellular region is the most overrepresented category in module 12, followed by the extracellular space and the extracellular vesicular exosome. From the 113 members of this module, 36 (32%) genes code for proteins in the extracellular region, which is a category with 1,525 genes, 8% of total number of human genes (adjusted p-value $\leq 7 \times 10^{-11}$).
Supplementary Figure 9. Expression and DNA-methylation of three genes from MMP family. *MMP9*, *MMP8*, and *MMP25* are the three genes from the matrix metalloproteinase (MMP) family that have relatively high contribution to our extracellular matrix eigengene. (a) They are significantly underexpressed in AML compared to MDS (adjusted p-values in the MILE dataset ≤ $10^{-53}$, $10^{-32}$, and $10^{-38}$, respectively.) (b) The y-axis shows the $\beta$ value, which corresponds to the percentage of DNA-methylation in each sample. For each gene, methylation at a locus close to its promoter is shown (Supplementary Data 1). These three genes are heavily methylated in the majority of 194 AML cases (red) in comparison to 368 control cases (blue), Welch’s t-test p-values are $10^{-138}$, $10^{-252}$, and $10^{-46}$, respectively.
Supplementary Figure 10. Robustness. To quantify the affect of noise on our analysis, we replaced random entries of the expression profile from the BCCA dataset with Gaussian noise, $\mathcal{N}(\mu, \rho)$, where $\mu = 0.008$ and $\rho = 0.95$ are the mean and the standard deviation of expression data, respectively. The x-axis shows the percentage of perturbed entries in the expression profile. The average accuracy of our decision tree over 1000 runs are shown on the y-axis. The error bars correspond to the standard deviation. The accuracy is very robust with respect to noise, for instance, even when 30% of the expression profile is perturbed, the decline in the accuracy is negligible (2%).
Supplementary Figure 11. Breast cancer survival analysis. Kaplan–Meier survival curves are shown for the three groups of ER+ patients classified by our survival analysis (Supplementary Note 4). Each plot corresponds to a dataset. We identified the biological signatures using METABRIC training dataset (a), and confirmed their predictive value in METABRIC validation (b) and MILLER (c) datasets (Methods). The low-risk patients are identified by regulated cell cycle and transcription (green). For this group, the probability of surviving more than 10 years is above 89% in all the three datasets. The p-values indicate that the difference between low and high risk groups is statistically significant.
**Supplementary Figure 12. Association with translational control.** The y-axis shows the p-value from overrepresentation analysis on the smaller module that was automatically-selected by breast cancer survival analysis. This module of 193 genes is significantly associated with translation and translational control.
Supplementary Note 4: Survival analysis on breast cancer. We applied a methodology similar to our AML-MDS analysis on METABRIC discovery dataset to identify 15 modules and compute the corresponding eigengenes. We used glmnet package (version 2.0-2) to fit a regularized Cox model with the Lasso penalty ($\alpha = 1$). The regularization path indicated that two modules with 319 and 193 genes are most associated with survival. We used the corresponding two eigengenes to fit an accelerated failure time model to the survival data. Specifically, we used `survreg` function from Survival package (version 2.38-3), Weibull distribution with `scale=1`, and the defaults values for the rest of the parameters. We used the fitted model to predict the survival time using only the two eigengenes. We chose two thresholds for the predicted values that maximized the precision of low and high risk predictions in the METABRIC discovery dataset. We inferred the two eigengenes in METABRIC validation and MILLER datasets to evaluate our accelerated failure time model. Using the same thresholds, our approach could identify low-risk patients in these two independent datasets with high specificity (> 89%, Table 3 and Supplementary Fig. 11). This illustrates the biological significance of the identified signatures.
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