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Making sense out of massive data by going beyond differential expression

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With the rapid growth of publicly available high-throughput transcriptomic data, there is increasing recognition that large sets of such data can be mined to better understand disease states and mechanisms. Prior gene expression analyses, both large and small, have been dichotomous in nature, in which phenotypes are compared using clearly defined controls. Such approaches may require arbitrary decisions about what are considered “normal” phenotypes, and what each phenotype should be compared to. Instead, we adopt a holistic approach in which we characterize phenotypes in the context of a myriad of tissues and diseases. We introduce scalable methods that associate expression patterns to phenotypes in order both to assign phenotype labels to new expression samples and to select phenotypically meaningful gene signatures. By using a nonparametric statistical approach, we identify signatures that are more precise than those from existing approaches and accurately reveal biological processes that are hidden in case vs. control studies. Employing a comprehensive perspective on expression, we show how metastasized tumor samples localize in the vicinity of the primary site counterparts and are overenriched for those phenotype labels. We find that our approach provides insights into the biological processes that underlie differences between tissues and diseases beyond those identified by traditional differential expression analyses. Finally, we provide an online resource (<http://concordia.csail.mit.edu>) for mapping users' gene expression samples onto the expression landscape of tissue and disease.

large-scale analysis | personalized medicine | phenotype classification

Although gene expression microarrays have been a standard, widely utilized biological assay for many years, we still lack a comprehensive understanding of the transcriptional relationships between various tissues and disease states. Even with the hundreds of thousands of expression array datasets available through public repositories such as National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus (1) (GEO), the lack of standardized nomenclature and annotation methods has made large-scale, multiphenotype analyses difficult. Thus, expression analyses have typically used the decade-old approach of comparing expression levels across two states (e.g., case vs. control) or a limited number of phenotype classes (2–4). Even recent large-scale gene expression investigations, whether they have attempted to elucidate phenotypic signals (5–7) or applied those signals for downstream analyses such as drug repurposing (8, 9), involve comparisons between two states or classes.

Comparative analyses, where transcriptional differences are directly measured between two phenotypes, inherently impose subjective decisions about what constitutes an appropriate control population. Importantly, such analyses are fundamentally limited in scope and cannot differentiate between biological processes that are unique to a particular phenotype or part of a larger process that is common to multiple phenotypes (e.g., a generic “cancer pathway”). Moreover, the results of such comparative analyses can be limited in generalizability as they make assumptions about the phenotypes being compared (10). Alternatively, in

a data-rich environment, we can take a holistic view of gene expression analyses.

In this paper we introduce scalable and robust statistical approaches that leverage the full expression space of a large diverse set of tissue and disease phenotypes to accurately perform and glean biological insights from both sample- and gene-centric analyses. By viewing a given phenotype in the context of this comprehensive transcriptomic landscape, we circumvent the need for predefined control groups and presupposed relationships between phenotypes (Fig. 1A). We devise, implement and validate the accuracy of an enrichment statistic that provides detailed phenotypic information for new samples when they are mapped onto and compared with the transcriptomic landscape (<http://concordia.csail.mit.edu>).

Our perspective on interpreting gene expression space helps uncover phenotype-specific marker genes beyond those discovered by traditional dichotomous views of gene expression. We introduce a method based on a finite impulse response filter (11) used in signal processing to reveal, for instance, marker genes involved in carbohydrate and lipid metabolism as key processes in breast cancer. Such findings are in contrast to those of traditional over- and underexpression based analyses, which focus on generic cancer processes not specific to breast cancer such as cell cycle and cell adhesion (12). Capitalizing on the hierarchical nature of the phenotypic labels associated with our samples, we also demonstrate that genes previously linked to specific types of carcinomas may actually be part of a broader “carcinoma” process. Finally, we illustrate how metastasized tumor samples are transcriptomically more proximal to other cancer samples from their respective primary sites, as opposed to cancerous tissue from the metastasis sites from which the samples were resected.

Results

Making Sense of the Transcriptomic Landscape. As an initial step towards a holistic approach to gene expression analysis, we must make sense of the substructure of the global transcriptomic landscape. We first constructed a curated gene expression database of 3,030 diverse samples (from 192 series) obtained from NCBI's GEO (1). These samples were annotated with their phenotypes (tissue of origin, disease state, etc.) using the anatomical and disease concepts in a custom subset of the Unified Medical Lan-

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Table 1. Concordia cross-validation performance on selected UMLS concepts

Concept	AUC	No. series	No. samples
Malignant neoplasms	0.82	74	855
Malignant neoplasm of breast	0.97	9	69
Malignant neoplasm of ovary	0.99	4	51
Malignant neoplasm of lung	0.97	4	98
Leukemia	0.99	13	151
Soft tissue	0.69	98	1,513
Breast	0.93	13	195
Ovary	0.95	8	103
Lung	0.95	9	131
Inflammatory disorder	0.79	13	91
Rheumatoid arthritis	0.93	7	31
Inflammatory bowel diseases	0.99	2	24

We see a significant increase in accuracy as more data is added to the underlying database. For example, when half of the samples associated with each concept are removed, the global performance is a mere 44%, compared to the aforementioned 93% (*Supporting Information*). This implies that the phenotypic signal becomes stronger and the power of this type of macroscopic analysis increases with the amount of underlying data. As our approach employs a nonparametric enrichment statistic that only requires the concept annotation of the samples in the original gene expression database, it can be updated in real-time without having to “retrain” the database. A system such as this could thus be deployed in a research or clinical setting where new samples are continually being added and analyzed, with minimal alteration of normal protocols.

Primed with the 3,030 labeled samples, we applied Concordia to 15,904 other GEO samples performed on the Affymetrix HG-U133 Plus 2.0 array. These enrichment scores represent the expression patterns as characterized by the 1,489 anatomy and disease-related concepts and can be used as an additional source of biological information when performing future large-scale gene expression analyses (*Supporting Information*).

Phenotypic-Specific Marker Genes. We developed a method to identify marker genes that characterize a specific phenotype in the context of broad transcriptomic landscapes, and not in the context of dichotomous classes. Instead of defining a marker gene as one that is over- or underexpressed in a case vs. control study using methods akin to t-tests, we define a marker gene as a gene that has a “localized” expression signature for a phenotype; i.e., how grouped together all of the samples are corresponding to that phenotype for that gene. If all of the samples for a phenotype have a very similar expression level (all high, all low, etc.), the gene may be considered a marker gene for that phenotype. We employ a finite impulse response filter (FIRF) (11) on each gene’s expression values across the entire database of 3,030 diverse expression samples to quantify the degree of expression level localization for a given phenotype. To generate the set of genes most relevant to a phenotype, we use the marker gene localization scores to rank all genes and then we identify the cutoff for the number of genes to include by balancing the set’s ability to accurately classify samples of its own phenotype while minimizing the presence of non-phenotype-specific signal (*Methods*). Not only does this method sidestep the requirement of defining appropriate “control” phenotype(s), it also facilitates the identification of thematically coherent gene signatures that reveal very different aspects of biology from traditional ones.

As an example, we derived the breast cancer gene set from a landscape of 673 samples representing 17 different cancerous tissues. The 74 genes that comprise this set are functionally enriched for processes related to breast-specific development, and carbohydrate and lipid metabolism (*Supporting Information*). These pathways, revealed through gene expression, are consistent

with independent clinical and genetic data suggesting an important role for carbohydrate and lipid metabolism in breast cancer. For example, women with type 2 diabetes may have higher susceptibility to breast cancer (16). Three genes specifically implicated in this analysis, *ENPPI*, *ADIPOQ*, and *PPARA*, are of particular interest. *ADIPOQ* is expressed in adipose tissue exclusively. Variants in the *ADIPOQ* gene and protein levels are implicated in prostate cancer (17) and breast cancer (18). Similarly, *ENPPI* levels have been correlated to progression-free survival in tamoxifen-treated patients with breast cancer (19). *PPARA* is one of a family of nuclear transcription factors that has been found to stimulate both adipocyte (fat cell) differentiation and fatty acid oxidation (20). Moreover, the *PPARA* signaling pathway has been implicated in breast cancer progression (21), and in a case-control study a polymorphism of *PPARA* was identified to be associated with a twofold increase in breast cancer (22).

Notably missing from this list of enriched pathways are processes commonly associated with cancer, such as cell-cycle and cell-adhesion (12). We can recreate this conventional perspective by selecting the set of candidate marker genes using a traditional permutation t-test-based method (*Methods*). This reveals enrichment for processes that are associated with cancer in general, but not specific to breast cancer, such as “cellular response to tumor necrosis factor,” “induction of apoptosis,” and other tumor-related processes (*Supporting Information*). Furthermore, according to the permutation t-test method, *PPARA* is less significant than nearly 17% of the other genes (*ADIPOQ* is in the top 2% and *ENPPI* is in the top 0.5%). In comparison, using the FIRF, the tumor-necrosis-related genes, such as *RIPK1*, *TRADD*, and *TNFRSF25*, do not appear until, respectively, 18%, 54%, and 97% of the other more breast-cancer-specific genes appear first.

To ascertain the “cancer” gene set using our FIRF-based method, however, we expanded the landscape of data to include not only 17 cancers but also 2,187 samples across 30 noncancerous tissue types. By comparing all cancers against all noncancers, we unsurprisingly then find that the most significant genes are functionally enriched for processes that are typically associated with tumors: “cell division,” “cell cycle,” and “DNA repair,” to name but a few. Taken together, landscape-based gene signature discovery can recapitulate canonical cancer pathways but also can identify a complementary set of gene signatures with distinct biological implications.

Specificity of Marker Genes. It has been suggested that the so-called “incidentalome” of incidental findings is a threat that has yet to be addressed in either biological or clinical settings (23). The consequences of noncomprehensive views of biomarkers, such as prostate-specific antigen, continue to cause needless harm and costs (24). By performing analyses in the context of a large database of biological samples, however, we see that many genes are not specific to a single disease.

To illustrate this, we took the 459 carcinoma samples in our database and computed the “carcinoma” marker gene localization scores by comparing them to the 270 other tumor samples. As the UMLS concepts are in a structured ontology, we computed the marker gene scores for the 13 concepts subordinate to “carcinoma” (e.g., “adenocarcinoma,” “adenosquamous carcinoma”) for which we had at least three expression samples. From the list of genes sorted by their carcinoma marker gene score p-value, we removed all genes that had a better p-value in any of the 13 subordinate concepts. This yielded a list of 5,805 genes that had better p-values at the more general concept “carcinoma” than at any of the more specific subordinate carcinoma types. Functional enrichment analyses of the top 10, 20, 50, 100, and 150 genes in this list reveals processes such as “regulation of cell adhesion,” “response to growth factors,” and other morphogenesis and development terms. Furthermore, within the sorted list of carcinoma genes, we see genes previously implicated in carci-

intermediate pathotypes that cross the boundaries of the conventional medical classifications. These intermediate pathotypes are more closely coupled to the actual underlying pathology, thus revealing not only shared pathology but also opportunities for development of shared treatment (30, 33). It may be the case that the expression signatures of diseases provide clues to a disease network (34) other than what classical medical knowledge dictates, thus providing insights to previously unknown disease relationships.

It has been proposed that the future of personalized medicine, and the proper application of genomic and genetic data, requires an understanding of both who the patient is and the characteristics of the subpopulation to which the patient belongs (35). Clinical applications of our approach, together with other genetic, environmental, and phenotypic information, could more accurately and consistently annotate clinical samples and provide an impartial view of the landscape of clinico-pathological classification. As we employ an enrichment statistic that only requires the usual standard of care in the labeling of samples, this system could be deployed in a clinical setting with minimal alteration of normal procedures. By shifting away from a dichotomous view and employing the global transcriptomic landscape, we hope to address one of the key requirements of personalized medicine and begin to answer one of its fundamental questions, “what other samples am I most similar to so that the most effective treatment can be administered?”

Methods

Normalizing the gene expression samples. Our database is comprised of 3,030 gene expression samples belonging to 192 series performed on the Affymetrix HG-U133 Plus 2.0 arrays that were obtained from NCBI's GEO (1). The original CEL files were downloaded from GEO and Microarray Suite (MAS) 5.0 normalized. Subsequently all probe-specific values were converted to gene-specific values using a trimmed mean. For the gene selection procedure, we log-normalized all of the expression values to be between -1 and 1 to ensure a normal distribution. For all of the other analyses, the expression values were additionally rank normalized.

UMLS Annotation. We follow the lead of Butte, et al. (36) and extracted the title, description, and source fields from each of the 3,030 expression samples and annotated them using the Java implementation of the National Library of Medicine's (NLM) MetaMap program, MMTx (37). A custom UMLS (13) thesaurus containing concepts from the UMLS, Medical Subject Heading, and Systematized Nomenclature of Medicine ontologies was generated using NLM's MetaMorphosis program. The automated annotations were manually verified and 672 UMLS concepts were kept. As these concepts only represented the most detailed level of annotation, they were mapped up the ontology such that a sample labeled with a specific concept also received labels corresponding to all of its ancestor concepts. Due to the domain of the data, we filtered the concepts to only those that are descendants of either “disease” or “anatomy,” resulting in 1,489 concepts.

Making Sense of the Transcriptomic Landscape. The transcriptomic landscape visualization is the first two PCs of the PC projection of the 3,030 centered and scaled gene expression samples. The phenotypic clusters portrayed by shaded regions were created by iteratively using the convex hull function (chull) in the R statistical language package. We performed the hierarchic analysis of the landscape by taking the 1,065 phenotypically normal samples in the soft-tissue cluster and recalculating the PCs. The convex hulls for the gastrointestinal and reproductive clusters were computed in the aforementioned fashion.

The tissue similarity network was generated by computing correlations of a representative sample of a tissue type to all other representatives of the other tissues. The representative was chosen to be the sample that was closest to the centroid in the set of samples for that phenotype. To contend with sampling bias, the correlations were computed 100 times, the centroid for each phenotype having been chosen from a random 75% subset of the samples for that phenotype. The network was then created based on the tissue-tissue relationships with an average correlation greater than 0.8 across all 100 subsampling runs. The colors of the nodes denote the general tissue class (blood, brain, gastrointestinal, reproductive, and other).

Our online resource also provides a visualization of where an input sample lies in the transcriptomic landscape. An input sample's coordinates are computed by centering and scaling its expression values by constants learned from the database and then applying the loadings from the first two PCs.

Picking Blood, Brain, and Soft Tissue-Specific Genes. Tissue-specific genes were selected by performing permutation t-tests comparing, for example, the log-normalized expression values for the blood samples for a given gene to the log-normalized expression values of the samples associated with brain and soft tissue. Each permutation run consisted of computing the t statistic for the actual labeling of the samples and comparing it to the t statistics produced when the labels were randomly permuted 200 times while keeping the sample size distribution constant. To counter the potential influence of sampling bias, this entire procedure was performed 100 times, each time using only a random 75% of the data for each tissue type. Genes with a false discovery rate corrected p-value of 0.05 or lower in all 100 runs were deemed significant. As there were genes with identical p-values, the genes were then sorted such that a gene with a larger difference in means between the phenotypes was ordered before those with a smaller difference. GO enrichment was performed on the top 50, 100, and 250 genes for each tissue type using FuncAssociate 2 (38). We report only the GO terms that had a resampling-based p-value less than 0.05.

Computing Phenotype-Specific Gene Signatures. To determine the level of localization of the expression intensities for a given gene, we employed a FIRF (11). For each gene g , phenotype p pair, we sort all of the expression samples by their expression intensities for g . Using a “sliding window” of size equal to the number of samples corresponding to p , we compute the fraction of samples in that window that are associated with p . The value is 1 if all samples in the window are associated with p and 0 if none of them are. This window is iteratively moved across the sorted list of samples to obtain a value for all positions. The marker gene score for a particular gene-phenotype pair is the maximum value that is achieved in any of the windows. A p-value is computed for each score using a binomial distribution.

To determine the appropriate cutoff for the number of genes to include in the gene set for phenotype p , the genes are first sorted according to their marker gene score from highest to lowest. We then iteratively examine the quality of the top n genes, balancing their positive predictive capability with the amount of additional noise. Starting with the first two highest scoring genes, we iteratively remove each sample s and compute its correlation to all other samples using only those two genes. We generate an ROC curve for s and use the AUC as a summary statistic. The ROC curve is generated by sorting all samples by their correlation to s and incrementing the true-positive count when that sample is associated with p and incrementing the false-positive count when that sample is not associated with p . Once all AUCs are computed for two genes, we add the next highest scoring gene and recompute all AUC values. We define the mean “hit” AUC as the average AUC obtained by all samples associated with p , and the mean “miss” AUC as the average AUC of all samples not associated with p . By taking the ratio of the mean hit AUC and mean miss AUC at each number of genes n , we determine the relevant set of genes as all genes in the sorted list up until the number of genes that maximizes this ratio.

To compare the performance of the FIRF to the traditional over- and underexpression-based analyses relying on differences in the mean expression levels in the phenotypes being studied, we performed a t-test for each gene and computed the empirical p-value based on 1,000 random permutations of the phenotype labels. As many of the p-values were 0 (or the same), we sorted the list of genes by the z score of the actual t statistic as compared to the 1,000 t statistics generated by the random permutations. GO enrichment was then performed using the Bioconductor GStats (39) library in R.

Enrichment Score Calculation. We use the database of gene expression samples to assess overenrichment for particular disease- and tissue-specific signals. Given a new expression profile, for each concept represented in the database, we calculate a statistic that measures the strength of association between the sample and concept, as implied by its similarity to the labeled database samples.

The statistic is calculated as follows. First, the database consisting of n curated expression samples $\{s_1, s_2, s_3, \dots, s_n\}$ is sorted (in decreasing order) according to each observation's Spearman correlation, ρ , with the new profile. Let $s_1, s_2, s_3, \dots, s_n$ represent the samples ordered according to their correlation coefficients $\rho_{s_1}, \rho_{s_2}, \rho_{s_3}, \dots, \rho_{s_n}$. For a given concept c in the set C , the set of all UMLS concepts in our database, let S_c be the set of all database samples associated with the concept. That is, $S_c = \{s_i | s_i \text{ is associated with } c\}$. We define an ordered list of x_i values:

$$x_i = \left(\frac{1 + \rho_{si}'}{2} \right) / \left(\sum_{s_j \in S_c} \frac{1 + \rho_{sj}'}{2} \right)$$

when sample s_j is associated with concept c , and

$$x_i = -1 / (n - |S_c|)$$

for all other samples that are not associated with concept c . Intuitively, when s_j is associated with the concept in question, the x_i value corresponds to the fraction of total correlation between the new sample and all database samples associated with the concept. All of the x_i values for the concept "hits" sum to 1, and all of the x_i values for the concept "misses" sum to -1 .

Then we compute a running sum of x_i across all n database samples and take the maximum value achieved by this running sum as our enrichment score (ES) for the concept in question:

$$\text{Enrichment Score}_c = \max_{1 \leq j \leq n} \sum_{1 \leq i \leq j} x_i$$

This sum across all n samples is zero. The concepts where there is strong positive deviation from 0 are the concepts whose associated samples are more highly correlated with the new profile than those samples that are not associated with the concept.

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Quantifying Performance. To quantify the ability of the method to recover UMLS concepts based on an input expression profile, we generate an ROC curve and calculate the AUC as a summary statistic for each concept represented in the database. To compute the ROC curve for each concept c in the database, we iteratively leave out each sample s and compute s 's enrichment score for c using the remaining database samples. We compute the running true-positive (TP) and false-positive (FP) counts by walking down the list of samples sorted by their enrichment score for c . The TP is incremented if the i th sample in the list is actually labeled with concept c . If the sample is not labeled with concept c , the FP is incremented. The true-positive results (TPRs) and FPRs are obtained by dividing TP and FP, respectively, by the number of known positives and negatives at each position i . By plotting the TPR vs. FPR we obtain the ROC curve. The larger the area under the ROC curve (AUC), the greater the gene expression signal for that concept as the samples with the highest enrichment scores for the concept were truly labeled with that concept.

When using this method to label a new sample, we compute its ES (w.r.t. the entire database) for each concept. We then report the system's estimated FPR for each concept at the sample's observed concept-specific enrichment score. These FPR values are derived from the running statistics used to generate the ROC plots: Look up the new sample's score position in the list of sorted scores and report the FPR at that position (if there is not an exact match, report the next-worst FPR).

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