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Application of Biostatistics and Bioinformatics Tools to Identify Putative Transcription Factor-Gene Regulatory Network of Ankylosing Spondylitis and Sarcoidosis

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Abstract

Transcription factors and corresponding *cis*-regulatory elements are considered key components in gene regulation. We combined biostatistics and bioinformatics tools to streamline identification of putative transcription factor-gene regulatory networks unique for two immune-mediated diseases, ankylosing spondylitis and sarcoidosis. After identifying differentially expressed genes from microarrays, we employed tightCluster to find tight clusters of potentially co-regulated genes. By subsequently applying bioinformatics tools to search for common *cis*-regulatory elements, putative transcription factor-gene regulatory networks were found. Recognition of these networks by applying this methodology could pave the way for new insights into disease pathogenesis.

Introduction

An oligonucleotide microarray experiment can provide rich information on gene expression patterns by simultaneously screening tens of thousands of transcripts from relatively a small quantity of solid tissue or blood. It has been used successfully to identify genes related to diseases such as lymphoma (Hans, et al., 2004), melanoma (Dai, et al., 2007), and breast cancer (Driouch, et al., 2007). Transcription factors and corresponding *cis*-regulatory elements, also called motifs, are considered key components in regulating gene expression patterns but continue to remain elusive because the binding sites are short sequence segments, scattered widely over the genomic non-coding regions, and these motifs are difficult to locate using

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Conflict of interest notice

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conventional approaches (Antes, et al., 2000; Barton, et al., 2001; Bonifer, 2000; Lee, et al., 2004; Michelson, 2002; Pennacchio and Rubin, 2001). With the complete sequence information for many eukaryotic genomes available and a publicly available online database of transcription factors, we can search for potential *cis*-regulatory elements more efficiently by combining biostatistics and bioinformatics methods for a group of co-regulated genes.

Since it is typical to detect hundreds or thousands of differentially expressed genes in a microarray experiment, it is essential to reduce the dimensions before applying any bioinformatics tools. Clustering analysis has been crucial in reducing the dimensions of microarray data analyses (Choi, et al., 2006; Fachin, et al., 2007; Nikpour, et al., 2007). Traditional clustering methods such as hierarchical clustering (Kaufman and Rousseeuw, 1990) and *k*-means clustering (Hartigan and Wong, 1978) have been used for detecting groups of genes that show similar expression profiles. However, these traditional clustering methods assign every gene to a cluster and require subjective decisions with regard to the number of clusters. This number may be difficult to predict when investigating complex biological processes although one can be guided by some post-hoc criteria that are based on homogeneity within clusters. Forcing all data points into a predetermined number of clusters could increase the possibility that a cluster contains multiple subclusters of genes that are regulated by different transcription factors. This might in turn increase noise and make identification of unique transcription factors for co-regulated genes difficult. A new generation of clustering algorithms has been proposed in recent years that can overcome these difficulties. These new methods do not require a pre-determined number of clusters nor do they force all genes to be grouped. Some examples are the tightClust (Tseng and Wong, 2005), split (Pena, et al., 2003), model-based clustering (mClust) (Fraley and Raphael, 2002), and dynamic tree cut (Langfelder, et al., 2008). The tightClust algorithm largely consists of two parts; a tight clustering step and a sequential search for stable clusters based on bootstrapping. One unique feature of tightClust is the inclusion of natural ranks of tightness of clusters because of sequential search. The split and mClust are based on a Gaussian mixture model for clustering. The split employs the jackknife-style prediction for clustering while the mClust uses the EM algorithm. The dynamic tree cut methods are novel algorithms that can detect clusters in a dendrogram from hierarchical clustering. For more details, refer to the original publication for each algorithm.

This study demonstrated a streamlined analysis for predicting putative transcription factor-gene regulatory networks by first utilizing one of the new clustering algorithms, tightClust (Tseng and Wong, 2005), to find tight clusters of differentially expressed genes between peripheral blood samples of ankylosing spondylitis and sarcoidosis patients and control groups, and then applying a bioinformatics tool, the Promoter Analysis and Interaction Network Toolset (PAINT) (Vadigepalli, et al., 2003) to identify putative transcription factor-gene regulatory networks based on 2000 upstream base pairs from the transcription start sites of genes in selected tight clusters.

Rheumatologists care for patients who suffer from inflammatory diseases induced by the immune system. Ankylosing spondylitis is among the most common diseases in most rheumatologic practices. Ankylosing spondylitis and its closely related conditions, including reactive arthritis, psoriatic arthritis, undifferentiated spondyloarthropathy, and the arthritis associated with inflammatory bowel disease affect greater than 1% of the population by many estimates (Sieper, et al., 2006). Although ankylosing spondylitis classically involves the sacroiliac joints, it can also affect other organs. For example, 40% of these patients develop eye inflammation, specifically anterior uveitis, which is synonymous with iritis (Brophy, et al., 2001). Sarcoidosis is less common in most rheumatologic practices because pulmonary disease is usually the predominant manifestation. Sarcoidosis, however, is an excellent comparator to ankylosing spondylitis because it is also immunologically mediated. In North America, ankylosing spondylitis and sarcoidosis rank first and second respectively as the two

most common systemic, immune-mediated diseases associated with uveitis (Rosenbaum, 1989). Thus far, there has been no report comparing gene expression profiles for these two diagnoses.

Microarray experiment and identifying significantly expressed genes

Peripheral blood samples were taken from recruited patients with ankylosing spondylitis or sarcoidosis attending a tertiary clinic at the Oregon Health & Science University (OHSU) for patients with uveitis, rheumatic disease, or lung disorders. Since medications can markedly affect gene expression, all blood samples were drawn from patients who were not receiving systemic corticosteroids or any sort of systemic immunomodulatory therapy at the time of blood draw. The control group was recruited from patients attending an ophthalmology clinic for routine eye care. These controls did not have a history of autoimmune disease, were not on oral corticosteroids or any immunomodulatory therapy, and had no evidence for uveitis on eye examination. There were 11 patients with ankylosing spondylitis, and 12 patients with sarcoidosis and 12 controls. The study received approval by the OHSU institutional review board.

The microarray experiment was performed by the Affymetrix Microarray Core in the OHSU Gene Microarray Shared Resource. RNA was amplified and labeled using the GeneChip Globin Reduction Protocol rev. 1 (Affymetrix, Inc., PreAnalytiX). Then, 10 µg of each labeled target were hybridized with a Human Genome U133 Plus 2.0 array (Affymetrix, Inc.) using standard protocols as described in the GeneChip Expression Analysis manual (www.affymetrix.com/support/technical/manual/expression_manual.affx). The U133 Plus 2.0 array contains 54,675 probe sets for over 47,000 human transcripts and variants. After hybridization, cell fluorescence intensity (CEL) files were imported into the R statistical language environment (R_Development_Core_Team, 2007) for normalization. We used the GC robust multi-array analysis (GC-RMA) developed by Wu and co-workers (Wu, et al., 2004) to correct background noise in perfect match (PM) probe data. Background corrected data were further normalized with the rank invariant probes proposed by Li and Wong (Li and Wong, 2001). The gene expression levels were summarized using a linear model estimated by the median polish algorithm (Irizarry, et al., 2003). After normalization, we applied the Significance Analysis of Microarrays (SAM) (Tusher, et al., 2001). This method is appropriate when the numbers of samples in a data set are small. In this method, testing yields the q-values (Storey and Tibshirani, 2003) for statistical significance, which are similar to the false discovery rate (FDR) (Benjamini and Hochberg, 1995) that controls for the expected ratio of false positives among those genes that exhibit significant levels of expression rather than for the false positives among non differentially expressed genes. For the computations, we used R and its add-on packages; “affy”, “gcrma” and “samr”.

Out of 54,675 probe sets, SAM identified 5139 probe sets as being statistically significantly different between groups with $q < 0.01$ based on robustified F-statistics implemented in SAM (Tusher, et al., 2001). This implies that about 13% of genes were differentially expressed taking multiple probe sets targeting the same gene into account. There were 487 probe sets that were upregulated only in the ankylosing spondylitis group, 881 probe sets upregulated only in the sarcoidosis patients and 11 probe sets upregulated in both diseases. Among them, there were probe sets for well-known inflammation related genes such as interleukin 1 receptor type I (IL1R1), interleukin 1 receptor type II (IL1R2) and interferon (alpha, beta and omega) receptor 1 (IFNAR1) upregulated in the ankylosing spondylitis group and signal transducer and activator of transcription (STAT1), intercellular adhesion molecule 1 (ICAM1) and interferon regulatory factor 5 (IRF5) upregulated in the sarcoidosis patients.

Descriptive comparisons of clustering algorithms

To find tight patterns in 5139 significantly changed probe sets, we employed the tightClust (Tseng and Wong, 2005), split (Pena, et al., 2003), model-based clustering (mClust)(Fraley and Raphael, 2002), and dynamic tree cut (Langelder, et al., 2008). For all methods, we decided to use default parameters due to the wide diversity of algorithms. For the tightClust, we tried three different target numbers of clusters; 25, 50 and 100. In addition, we tried both dynamic tree methods proposed by Langelder and the coauthors (Langelder, et al., 2008).

Table 1 shows descriptive statistics of clustering results. All algorithms except mClust grouped about 44% ~ 76% of data in 7 ~ 98 clusters. From Table 1, tightClust with target number of clusters 50 and 100, tightClust(50) and tightClust(100), returned smaller clusters than other methods. Note that for some algorithms we standardized data within a probe set. Both mClust and split utilized a mixture of Gaussian distributions and produced different results when data were standardized within probe sets. In addition, the results of dynamic tree cut methods were different depending on the choice of linkage method in hierarchical clustering. We used the average linkage.

Putative transcription factor-gene regulatory networks by PAINT and TRANSFAC

The results from tightClust(50) and tightClust(100) were further analyzed by using PAINT v3.5 (Vadigepalli, et al., 2003) in conjunction with the TRANSFAC public database (<http://www.gene-regulation.com/>) to identify putative transcription factor-gene regulatory networks. The PAINT fetches upstream DNA sequences of genes and passes the sequences to TRANSFAC database search algorithm for known motif sequences in the TRANSFAC database to identify enriched putative common motifs across genes. Since PAINT could not identify any enriched transcription factors for the majority of small clusters from tightClust (100), the results from tightClust(50) will be reported hereafter.

There were 10 clusters that included significantly upregulated transcripts in the ankylosing spondylitis group and 19 clusters that contained significantly upregulated transcripts for the sarcoidosis group. The rest consisted of significantly downregulated genes in either group. Figure 1(a) – (f) shows the heat maps of the top 3 upregulated clusters in each disease.

All 29 tight clusters with upregulated transcripts in one of the diseases were further analyzed by using PAINT (Vadigepalli, et al., 2003) in conjunction with TRANSFAC public database (<http://www.gene-regulation.com/>) for transcription factors within 2000 base pairs upstream from the transcription start sites after combining entries when there were multiple probe sets for a transcript and removing entries for unknown transcripts. Table 2 summarizes common transcription factors for transcripts in the top 3 tight clusters of each disease that were significantly more frequent in each cluster compared to the other transcription factors of the human genome. Given the exploratory nature and small to moderate sizes of clusters, we used the marginal p-value < 0.05 to define significance. Figure 2(a) – (f) shows the corresponding potential transcription factor regulatory networks with related genes. These results show that there are distinct differences in the putative transcription factor regulatory profiles of ankylosing spondylitis and sarcoidosis. Among the top tight clusters, many immune-related genes were found in the second and third clusters of ankylosing spondylitis as well as in the first cluster of sarcoidosis. In particular, cluster 3 of the ankylosing spondylitis group, Figure 2(c), contained several genes with motifs for the CDP/CR1 transcription factor that regulates immune responses. In addition, we also found a strong STAT1 signature in one of the upregulated clusters for the sarcoidosis group. The biological implication of these findings is under review elsewhere or in preparation as it is beyond the scope of this manuscript.

Discussion

By combining tightClust with the bioinformatics tools, PAINT and TRANSFAC database, the process of making predictions for putative transcription factor regulatory mechanisms was streamlined and the potential differences in the regulatory networks were shown for two immune-mediated diseases, ankylosing spondylitis and sarcoidosis. Figure 3 illustrates the analysis flow used in this study. This is a significant step forward from a descriptive gene expression pattern analysis. This approach could reveal differences in the pathogenesis of ankylosing spondylitis and sarcoidosis and thus lead to new diagnostic, prognostic or therapeutic approaches. These predicted regulatory networks will require further verification by using other independent samples and confirmatory biological experiments.

There can be other clustering algorithms that are designed to group only tight data points since this is a hot research topic in microarray studies. One example is adaptive quality-based clustering by De Smet et al. (De Smet, et al., 2002). It is a heuristic iterative two-step method which has been proposed specifically for microarray data with the similar aim of clustering only tight data points. However, we could not finish the adaptive quality-based clustering due to a processing error at the time of analysis.

Because development of the clustering algorithms that we tested was based on different methods or models, their performance assessment in terms of revealing common transcription factors is not straightforward. In addition, transcription factors are an active research topic and the TRANSFAC database is constantly growing. As our knowledge grows and our models evolve, the available algorithms will need periodic reevaluation.

Care must be taken when applying these tools. As with any analysis of these hybridization microarray assays, normalization of the values among arrays is critical. The current results from tightClust were based on tight clustering with the standardization of gene expression within a probe set. The results without standardization were different and generally expression patterns within a cluster were not consistent since the tightClust used *k*-means clustering in each iteration of resampling. We also observed that the average number of genes per cluster was inversely proportional to a choice of target number of clusters. We tried 25, 50 and 100 while other parameters were the same. The median numbers of genes per cluster were 114, 45, and 40 with a total of 3267, 2487, and 2248 genes clustered, respectively. It is not clear whether this property is of concern. Having too many genes and too few genes in a cluster would both impede our ability to identify associations, such as regulatory networks, among the cluster members. In addition, since new information is frequently added to transcription factor databases, a future analysis may reveal other regulatory networks. Nonetheless, the tools described here provide a useful aid to generating plausible hypotheses to be tested in a biologic system. Although small tight clustering increases the possibility of artifactual groupings, the results make predictions of transcription factor-gene regulatory networks more efficient and are more readily analyzed for biochemical and regulatory pathways as demonstrated here for transcription factor-based networks.

Current therapy for either ankylosing spondylitis or sarcoidosis lacks consistent efficacy. For example, while tumor necrosis factor inhibitors are proving highly effective in the treatment of ankylosing spondylitis, roughly 20 to 40% of patients demonstrate an inadequate clinical response (Davis, et al., 2007). Pharmacological techniques are rapidly emerging to allow therapeutic targeting of transcription factors and their binding sites. Such a therapeutic approach has the danger of being more toxic than the targeting of a specific cytokine, but a corollary to this is that this approach potentially will have much greater therapeutic efficacy compared to the inhibition of a specific cytokine or inflammatory mediator. The identification

of clusters of upregulated transcripts should lead to novel therapeutic approaches and new insights into disease pathogenesis.

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Figure 1.

Heat maps for the top three tightest clusters of upregulated transcripts in patients with ankylosing spondylitis (AS) or sarcoidosis.

White: upregulated; Black: downregulated. a1 = AS patient 1; s1=sarcoidosis patient 1; c1=control 1.

- (a) Cluster 1 for AS
- (b) Cluster 2 for AS
- (c) Cluster 3 for AS
- (d) Cluster 1 for sarcoidosis
- (e) Cluster 2 for sarcoidosis
- (f) Cluster 3 for sarcoidosis

Brn-2/V\$BRN2_01
 AhR/Ant/V\$AHRARNT_01
 HLF/V\$HLF_01

571 (BACH1)
 79730 (FLJ14001)
 192670 (EIF2C4)
 4783 (NFIL3)
 57458 (TMCC3)
 5728 (PTENP1)
 11191 (PTENP1)

HIF-4/V β HIF4_01
 CRE-BP1/V β CREBP1_01
 Tax/CREB/V β TAXCREB_02

10335 (MRVI1)
 399716 (DKFZp667F0711)
 604 (BCL6)
 10335 (MRVI1)
 50807 (DDEF1)
 116496 (Clorf24)
 6556 (SLC11A1)
 9537 (TP53I11)
 10154 (PLXNC1)
 6583 (SLC22A4)
 8301 (PICALM)

NF-Y/V\$NFY_C
 Tax/CREB/V\$TAXCREB_01
 HEI1/V\$HEI1_01
 CDP CR1/V\$CDPCR1_01

7421 (VDR)
 2212 (FCGR2A)
 4851 (NOTCH1)
 121260 (SLC15A4)
 9846 (GAB2)
 5509 (PPP1R3D)

Freac-4/V\$FREAC4_01		150000 (ABCC13)
XBP-1/V\$XBP1_01		1508 (EEF1G)
MEF-2/V\$MEF2_03		8780 (RIOK3)
HKX2-5/V\$HKX25_01		8727 (RAD21)
CP2/V\$CP2_01		2235 (FECH)
ELK-1/V\$ELK1_01		7111 (TMOD1)
		8395 (PIP5K1B)
		11163 (NUDT4)
		11163 (NUDT4)
		7504 (XK)
		2994 (GYPA)
		2994 (GYPA)
		26994 (RNF11)
		26994 (RNF11)
		8724 (SNX3)
		2993 (GYPA)
		2993 (GYPA)
		63893 (UBE2O)
		51327 (ERAF)
		80127 (C14orf45)
		81689 (HBLD2)
		665 (BNIP3L)
		665 (BNIP3L)
		57095 (C1orf128)

Figure 2.

Predicted transcription factor regulatory networks for the top 3 tight clusters for ankylosing spondylitis or sarcoidosis by PAINT: Promoter Analysis and Interaction Network Toolset in conjunction with TRANSFAC Public database. Column headings are transcription factor names and row headings are gene symbols with Entrez gene IDs. A grey square means a transcription factor binding sites exists in a gene whereas a black square indicates that a transcription factor binding sequence was not found.

- (a) Cluster 1 of upregulated in ankylosing spondylitis
- (b) Cluster 2 of upregulated in ankylosing spondylitis
- (c) Cluster 3 of upregulated in ankylosing spondylitis
- (d) Cluster 1 of upregulated in sarcoidosis
- (e) Cluster 2 of upregulated in sarcoidosis
- (f) Cluster 3 of upregulated in sarcoidosis

Figure 3. Streamlined analysis flow to predict putative transcription factor-gene regulatory networks. The specific methods used in this manuscript are indicated in parentheses.

Table 1

Descriptive statistics of clustering results by various algorithms for 5139 differentially expressed probe sets. Numbers in tightClust() are target numbers of clusters. In mClust, numbers of genes are actual sizes of four clusters. All numbers are rounded-off at the decimal point.

Cluster	Data standardized within a probe set	Number of clusters	Size of clusters (number of probe sets)				Number of probe sets clustered out of n=5139 (%)	Number unclustered
			Min.	1 st Quartile	Median	3 rd Quartile	Max.	
tightClust (25)	y	25	64	92	114	129	358	1872
tightClust (50)	y	50	17	32	45	61	134	2652
tightClust (100)	y	98	9	16	20	28	70	2891
Split	n	25	49	61	75	165	652	1216
mClust	n	4	700	971	-	1687	1781	0
Dynamic tree	y/n	7	97	112	204	312	976	2814
Dynamic hybrid	y/n	21	30	51	100	142	380	2699

Enriched common transcription factors within upstream 2000 base pairs from transcription start sites in top 3 tight clusters of ankylosing spondylitis and sarcoidosis groups.

Table 2

Ankylosing spondylitis				Sarcoidosis		
Cluster 1	Cluster 2	Cluster 3		Cluster 1	Cluster 2	Cluster 3
HLF	HNF-4	CDPCR1		Nkx2-5	USF	Hand1
Brn-2	Tax/CREB	HEN1		CP2	GATA-1	CRE-BP1/c-Jun
Ahr/Amt	CRE-BP1	Tax/CREB		Elk-1	c-Myc	CRE-BP1
		NF-Y		Freac-4	CREB/V	CREB
				XBP-1	CRE-BP1	R
				MEF-2	CRE-BP1/c-Jun	E2
					NRF-2	Clox
						MEIS1A
						MEIS1B