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Using the *attract* method to identify core pathways in juvenile idiopathic arthritis

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ABSTRACT. The aim of this study was to identify core pathways associated with juvenile idiopathic arthritis (JIA) using the *attract* method. Kyoto Encyclopedia of Genes and Genomes pathways were determined using the GSEA-ANOVA method, based on the gene expression data of JIA. Syn-expression groups within core attractor pathways were identified by hierarchical clustering. Correlated sets of genes exhibiting highly similar profiles to the syn-expression groups were identified and each correlated set was subjected to a gene ontology functional enrichment analysis to discover potentially shared biological themes. Based on a false-discovery rate < 0.05, we identified 11 significant pathways were identified as potential attractors. Flag genes

or uninformative genes were removed and 5 discriminative pathways: the proteasome, ribosome, protein export, spliceosome, and Parkinson's disease pathways were identified. A final set of syn-expression groups with a consistent trend of relative expression of pathway-related genes was obtained; that is, the proteasome, ribosome, protein export, spliceosome, and Parkinson's disease pathways were composed of 2, 2, 1, 2, and 3 clusters, respectively. Genes in each correlated set shared common roles, and changes at the pathway level were more likely to be real. In light of these, the *attract* method was able to on expand important context to find distinguishing expression patterns within pathways. This paper predicted that the functional themes involved in protein synthesis (such as proteasome, ribosome, spliceosome) were closely related to the progression of JIA, which might contribute to the detection of therapy target for JIA.

Key words: Juvenile idiopathic arthritis; *Attract*; Syn-expression group; Gene ontology

INTRODUCTION

Juvenile idiopathic arthritis (JIA), characterized by swelling or pain in the joints, or limitation in joint movement, is a non-infective, autoimmune inflammatory joint disorder affecting children aged <16 years for a duration of 6 weeks or more (Ravelli and Martini, 2007). The clinical heterogeneity of this disease is responsible for its varying courses and outcomes, such as self-limited arthritis with no long-term disability and destructive arthritis with severe disabilities (Lovell, 2006). Despite the recent advances in the treatment of JIA, its recurrence rate remains high, with >40% of all patients carrying the disease over to adulthood (Bertilsson et al., 2013). Polyarticular JIA is a major subset of JIA involving a large number of joints, and with a tendency to worsen over time; therefore, this disease can lead to serious complications if left untreated (Davidson, 2000), specifically during early stages of the disease. However, lack of an effective and specific approach hinders the early diagnosis of polyarticular JIA. Therefore, a better understanding of the pathogenesis of JIA might help in the development of effective methods for early diagnosis and the identification of underlying therapeutic targets.

JIA is an autoimmune disorder whose pathogenesis is affected by various environmental and genetic factors. A previous study has indicated that interleukin-1 plays an important role in mediating the inflammatory cascade underlying systemic onset JIA (Pascual et al., 2005). Moreover, polymorphisms in the *UNC13D* gene are believed to contribute to the development of systemic JIA (Hazen et al., 2008). In contrast, Tang et al. (2014) proved the lack of any correlation between polymorphisms in interferon regulatory factor-5 and JIA susceptibility, contradictory to the results seen in other autoimmune diseases. Genes are strictly regulated in cells to execute proper biological functions in response to the perturbations caused by phenotypic changes. Pathway analysis is currently the first-choice method to extract and explain the underlying biology of genes with decreased complexity and increased explanatory power.

Generally, significant genes exhibiting differential expression patterns between different conditions are identified using expression-based analytical methods, followed by a

post hoc application of knowledge (obtained from databases such as the Kyoto Encyclopedia of Genes and Genomes; KEGG) to identify potential functional interpretations (Kanehisa and Goto, 2000). Although this method can be used to annotate large datasets, several genes with patterns that are significantly correlated to the selected genes are not identified because of the non-inclusion of KEGG resources. Fortunately, *attract*, an approach described by Mar et al. (2011a), can expand on the context that is not considered in the traditional KEGG method. Moreover, *attract* has a larger impact on the pathway number, as well as the pathway relevance.

In this study, core pathways associated with the development and progression of JIA were identified using the *attract* method, by detecting coordinated genome-wide changes in the gene expression. This method can be summarized in the following four steps: identification of the core KEGG pathways with considerable differences in gene expression between JIA patients and normal subjects; screening of syn-expression groups within a core attractor pathway module; identification of gene sets that exhibit profiles that are highly similar to the syn-expression groups within a core attractor pathway module; and the isolation of potentially shared biological processes by functional enrichment analysis of each of the syn-expression groups. Here, we attempted to demonstrate the power of the *attract* method in extracting the genome-wide expression data of JIA, and in identifying the underlying mechanisms and therapeutic targets of JIA.

MATERIAL AND METHODS

Microarray data

In this study, genome-wide peripheral blood gene expression data in children with polyarticular JIA (Accession No.: E-GEOD-13849) was downloaded from the ArrayExpress database, based on the GPL570 platform of the [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The dataset E-GEOD-13849 was composed of samples obtained from 59 healthy children and 61 children with polyarticular JIA. The sample characteristics are presented in a previous study (Griffin et al., 2009). A total of 54,675 probes were obtained for subsequent analyses.

Attractor analysis

The *attract* method was employed to identify the core pathways in JIA (Mar, et al., 2011a). This method is essentially the inverse of traditional gene expression analysis approaches, and is comprised of four key steps: determining the core KEGG pathways showing significant differential expression between JIA and normal conditions; identifying different syn-expression groups within a core attractor pathway module; finding sets of genes that exhibit highly similar profiles to the syn-expression groups in an attractor pathway module; and functional enrichment analysis of each syn-expression group to discover potentially shared biological themes.

KEGG pathway

The core pathway modules were defined by KEGG and identified by GSEA-ANOVA,

which identified pathways showing the most differential expression changes between JIA and normal conditions. Briefly, 54,675 probes were subjected to the KEGG enrichment analysis, and were assigned to one or more KEGG pathways. Pathways with less than 5 probes were discarded and the pathway level was examined using GSEA-ANOVA, a gene set enrichment algorithm based on ANOVA.

In this approach, ANOVA model was fitted to each gene and the gene expression was modeled via a single factor representing the groups as different levels of this class. The *F*-statistics was computed for all genes using the ANOVA model. In this study, the *F*-statistic reflects the strength of association of the expression of a gene between JIA and normal conditions. That is, a large *F*-statistic was indicative of strong condition-specific changes, while small *F*-statistic exhibited minimal condition-specific expression changes. The informative genes were then identified based on the *F*-statistics.

Subsequently, a KEGG pathway enrichment analysis was performed using probes on the array. As a large *F*-statistic is indicative of strong condition-specific changes, a pathway comprising gene members with a large *F*-statistic presented accumulated condition-specific changes. This relationship was verified using a *t*-test, wherein a comparative analysis of $\log_2$-transformed *F*-statistics between the pathway distribution and global distribution was implemented. The resulting P values were adjusted using the Benjamini-Hochberg false discovery rate (FDR)-based method (Benjamini and Hochberg, 1995), and KEGG pathways with FDR < 0.05 were classified as attractors.

Syn-expression groups

In this step, each remarkable and discriminative attractor pathway was decomposed into subsets of genes that shared similar expression patterns named “syn-expression groups”, which was the name originally used by Niehrs and Pollet (1999). A syn-expression group is a cluster of functionally interacting genes whose expression is tightly coordinated, which could serve as a discriminating profile to summarize the differential expression across two conditions. Moreover, it might be a key determinant factor leading to disease development. Therefore, in this study, we identified the syn-expression groups by decomposing significant pathways into correlated subsets. Briefly, the LIMMA model was employed to remove genes exhibiting no significant changes in expression between the two conditions. The remaining genes were subjected to a hierarchical clustering model, based on the Pearson correlation coefficient distance measure, to decompose each significant pathway into correlated subsets or syn-expression groups. The optimal number of syn-expression groups was decided via an informativeness metric (Mar et al., 2011b). The average expression profiles of the syn-expression groups were then analyzed.

Correlated partners of syn-expression groups

In this approach, the core attractor pathway modules and the syn-expression groups were deduced from information restricted to the KEGG sources. Consequently, these inferences were of high quality. However, this accuracy came at the expense of low coverage, as only a small proportion of the genome was ultimately described. Significantly, all genes with highly correlated patterns within the original data were extrapolated using the syn-expression

groups. Correlation coefficients between genes annotated to the syn-expression group and unannotated genes were calculated in each syn-expression group. In this study, a cutoff value of 0.85 was used to identify correlated partners of syn-expression groups on the chip that shared similar expression patterns to the syn-expression groups. Finally, correlated gene sets were constructed, which extended the analysis to the entire expression dataset by identifying genes highly correlated to the discriminating patterns (syn-expression groups).

Functional enrichment analysis

In this study, gene ontology (GO) functional analysis was implemented for each correlated partner to identify trends in common functions potentially shared by these genes. Significant terms were identified based on the following criteria: FDR < 0.05 and counts >10.

RESULTS

Identification of attractor pathway modules

Based on the gene expression data obtained from healthy and polyarticular JIA samples, the core attractor pathways whose expression differed between the two groups were identified using the *attract* method. A total of 11 significantly enriched pathways with a threshold FDR value < 0.05, believed to be attractors, were screened out, as depicted in Table 1.

Table 1. Significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified by *attract* that discriminate between two groups based on false discovery rates (FDR) < 0.05.

KEGG ID	KEGG term	FDR	Number of detected genes
3050	Proteasome	1.96E - 07	75
4080	Neuroactive ligand-receptor interaction	1.24E - 05	508
3010	Ribosome	4.43E - 05	141
982	Drug metabolism - cytochrome P450	1.14E - 03	119
3060	Protein export	1.14E - 03	51
3040	Spliceosome	5.47E - 03	287
3018	RNA degradation	1.36E - 02	174
5012	Parkinson's disease	1.85E - 02	228
4740	Olfactory transduction	3.16E - 02	173
190	Oxidative phosphorylation	3.68E - 02	232
4020	Calcium signaling pathway	3.85E - 02	467

Identifying syn-expression groups

Following the identification of candidate pathways, we categorized pathway-defined gene lists into highly correlated subgroups to highlight syn-expression groups reflecting gene sets responsible for the condition-specific differences. The informativeness metric was used to determine the optimal number of clusters. Removal of the flag genes or uninformative genes resulted in the isolation of 5 discriminative pathways (proteasome, ribosome, protein export, spliceosome, and Parkinson's disease). The syn-expression groups were isolated by decomposing each of these 5 significant, discriminative pathways into correlated subsets via hierarchical clustering. For example, the proteasome pathway was composed of 2 syn-

expression groups, which were composed of 11 and 6 correlated genes (Figure 1). Similarly, 2 syn-expression groups were identified for the ribosome pathway, composed of 24 and 8 correlated genes (Figure 2). The protein export pathway, however, was composed of only 1 syn-expression group, comprising 17 correlated genes (Figure 3). We screened 2 syn-expression groups for the spliceosome pathway, comprising 11 and 47 correlated genes (Figure 4). Finally, we isolated 3 syn-expression groups from the Parkinson's disease pathways, comprising 6, 10, and 38 correlated subsets of genes (Figure 5). Accordingly, we found that the trend of the relative expression of pathway-related genes in these syn-expression groups was consistent. Therefore, we obtained unique syn-expression groups from each significant pathway, which reflected the pathway-specific expression patterns driven by a few genes.

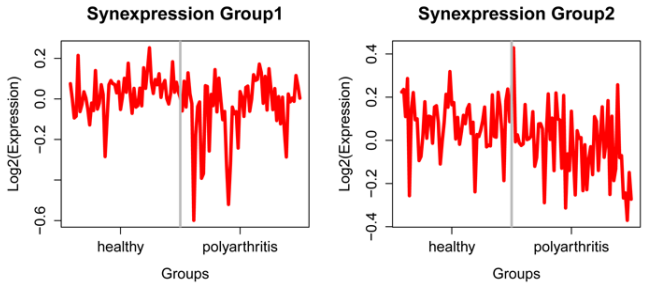

Figure 1. Average expression profiles of the syn-expression groups for the proteasome pathway. Sample categories are listed across the X-axis and the $\log_2$ (expression) is presented across the Y-axis. Each inflection point represents the average gene expression in each sample within a group.

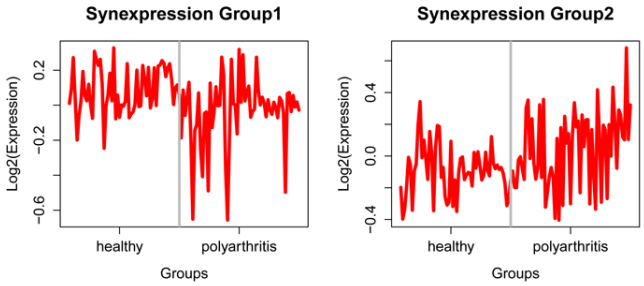

Figure 2. Average expression profiles of the syn-expression groups for the ribosome pathway.

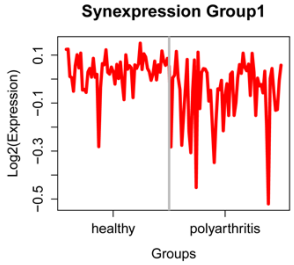

Figure 3. Average expression profiles of the syn-expression groups for the protein export pathway.

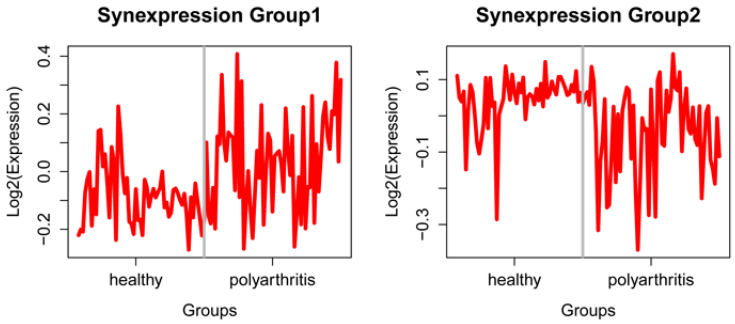

Figure 4. Average expression profiles of the syn-expression groups for the spliceosome pathway.

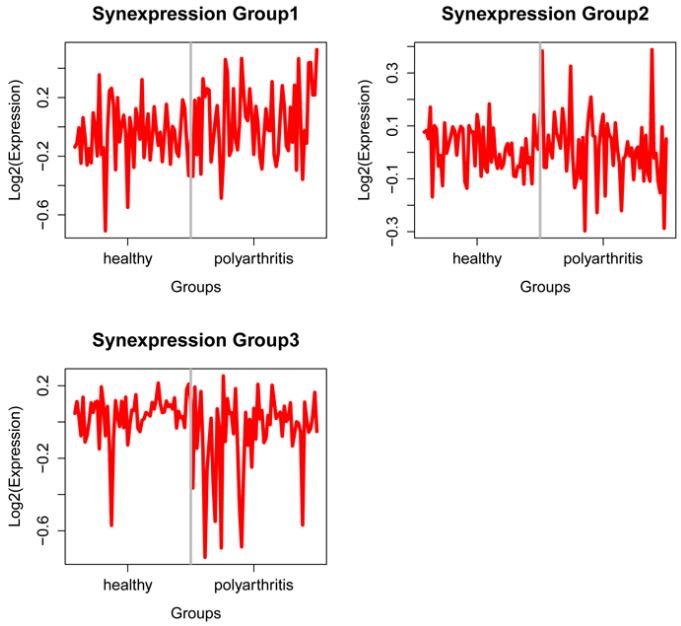

Figure 5. Average expression profiles of the syn-expression groups for pathways specific for Parkinson's disease.

Identification of correlated partners of syn-expression groups

In the initial GSEA steps of our study, genes with highly correlated patterns to the syn-expression groups were not included because of the lack of inclusion in KEGG resources. However, under a cutoff value (correlation coefficient) of 0.85, we identified 2, 2, 1, 2, and 3 clusters for the proteasome, ribosome, protein export, spliceosome, and Parkinson's disease pathways, respectively. Moreover, we discovered a total of 18, 72, 5, 51, and 161 unannotated genes for these pathways, respectively (see **Figures S1**, **S2**, **S3**, **S4**, and **S5**). Therefore, the *attract* method was able to expand important context, which was not seen in the traditional KEGG method.

GO functional enrichment analysis

Significantly enriched GO terms with FDR < 0.05 and counts >10 were identified for all pathways: 16 significant GO terms were identified in the proteasome pathway; genes were enriched in 22 significant functional terms in the ribosome pathway; and 20, 27, and 34 GO categories were identified for the protein export, spliceosome, and Parkinson's disease pathways, respectively. Of note, the two most significant GO terms of genes in correlated sets of the spliceosome pathway were catalytic step 2 spliceosome and spliceosomal complex. The genes in each correlated set shared common roles; accordingly, these genes belonged to the same GO term, and changes at the pathway level were more likely to be real.

DISCUSSION

In this study, the *attract* method was used to identify core pathways to explore the underlying molecular mechanism of JIA. Five core discriminative pathways (proteasome, ribosome, protein export, spliceosome, and Parkinson's disease pathways) were screened out, and the uniqueness of the syn-expression groups obtained from each core pathway, which reflected the pathway-specific expression patterns driven by a few genes, was confirmed. Accordingly, the genes in each syn-expression group belonged to the same GO term, which seemed to verify the authenticity of the pathway-level changes.

Expression-based analysis was developed to screen differentially expressed genes between experimental groups (Reiner et al., 2003). Subsequently, KEGG or meta-analysis is used to identify the underlying functions of the discovered genes. On the other hand, several genes might be ignored because of the lack of inclusion in the KEGG. Fortunately, *attract*, a modular process described by Mar et al. (2011a), has been shown to expand on important context that is not seen in traditional KEGG analyses. Moreover, *attract* has a larger impact on the pathway number, as well as the pathway relevance. *Attract* expands these deductions on these bases by identifying newly regulated coordinating genes that may be related to the mechanisms of disease. In agreement with this, expression-based analysis isolated only those correlated gene sets that exhibit profiles similar to the syn-expression groups; for example, *PPIB*, which is not a proteasome pathway-related gene, was not extracted. Therefore, we inferred that the *attract* method could help in the identification of crucial pathways and genes that could help uncover the disease mechanisms.

In this study, the proteasome pathway was identified as a core pathway of JIA using the *attract* method. Proteasomes, which form a major part of the ubiquitin-proteasome system (UPS), regulate several enzymatic activities that are responsible for controlling the gene expression and gene-environment interaction (Wang et al., 2007; Konstantinova et al., 2008). Specifically, insufficient proteasome function has also been reported to contribute to the pathophysiology of inflammatory disease (Zemeckienė et al., 2013). UPS (comprising ubiquitin and proteasome) has been shown to mediate the processing and degradation of a majority of regulatory proteins in eukaryotes. Moreover, UPS is the primary factor that activates the NF-κB signaling pathway (Hershko, 2005), which has been shown to play a major role in arthritis progression in animal models of inflammatory arthritis (Mor et al., 2005). Specifically, proteasome inhibitors are believed to inhibit NF-κB activity (Jana, 2008). However, proteasome inhibitors have been shown to induce osteoclast differentiation and

activate bone erosion, resulting in the development of chronic arthritis (Romas et al., 2000; Polzer et al., 2011). Accordingly, the proteasome pathway was theorized to play a core roles in JIA, probably by way of NF-κB signaling pathway regulation and bone destruction.

Attract also identified the spliceosome pathway as a significant pathway in JIA. Alternative splicing of genes is a fundamental and significant mechanism responsible for the regulation of gene function, and plays an important role in disease occurrence and development (Modrek et al., 2001). JIA is a well-known autoimmune and inflammatory joint disorder. Previous studies have reported a connection between the spliceosome-mediated autoimmune response and the development of rheumatoid arthritis (Hassfeld et al., 1995). In fact, Heinhuis et al. (2011) reported that splicing of IL-32γ into IL-32β exerts a protective role against inflammatory arthritis, by dampening the secretion of IL-32γ, a potent cytokine responsible for enhanced inflammatory arthritis. Moreover, GO functional enrichment of genes in correlated sets, performed to verify the *attract* method, identified catalytic step 2 spliceosome and spliceosomal complex as the most significant GO terms. This strongly suggested that *attract* could be used to expand the significant context, and that the spliceosome pathway plays a major role in JIA by regulating the expression of inflammatory factors.

This study is subject to several drawbacks: the sample size was not large enough to affect the conclusions to a certain degree; the results obtained by bioinformatic analyses were not verified *in vivo*. However, despite these disadvantages, we believe that the *attract* method and the predicted core pathways (proteasome and spliceosome pathways) offer investigators with valuable resources to better understand the mechanisms of JIA, to detect biomarkers of novel underlying pathways, and identify drug targets for JIA therapy.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary material

Figure S1. Average expression profiles of the correlated set of genes in the proteasome pathway. Gene clusters 1 and 2 were composed of 18 and 0 correlated genes, respectively. The X- and Y-axes listed the sample categories and $\log_2$ (expression), respectively. Each inflection point reflects the average gene expression of each sample within a group.

Figure S2. Average expression profiles of the correlated set of genes in the ribosome pathway. Gene clusters 1 and 2 were composed of 72 and 0 correlated genes, respectively.

Figure S3. Average expression profiles of the correlated set of genes in the protein export pathway. The cluster was composed of 5 correlated genes.

Figure S4. Average expression profiles of the correlated sets of genes in the splicesome pathway. The clusters 1 and 2 were composed of 0 and 51 correlated genes, respectively.

Figure S5. Average expression profiles of the correlated sets of genes in the Parkinson's disease pathways. Gene clusters 1, 2, and 3 were composed of 62, 0, and 99 correlated genes, respectively.