A Network-Based Analysis of Traditional Chinese Medicine Cold and Hot Patterns in Rheumatoid Arthritis

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Abstract:

Objective- Rheumatoid arthritis (RA) is a heterogeneous disease, and traditional Chinese medicine (TCM) can be used to classify RA into different patterns such as cold and hot based on its clinical manifestations. The aim of this study was to investigate potential network-based biomarkers for RA with either a cold or a hot pattern.

Method- Microarray technology was used to reveal gene expression profiles in $CD4^+$ T cells from 21 RA patients with cold pattern and 12 with hot pattern. A *T*-test was used to identify significant differences in gene expression among RA patients with either cold or hot pattern. Cytoscape software was used to search the existing literature and databases for protein–protein interaction information for genes of interest that were identified from this analysis. The IPCA algorithm was used to detect highly connected regions for inferring significant complexes or pathways in this protein–protein interaction network. Significant pathways and functions were extracted from these subnetworks by the Biological Network Gene Ontology tool.

Result- Four genes were expressed at higher levels in RA patients with cold pattern than in patients with hot pattern, and 21 genes had lower levels of expression. Protein–protein interaction network analysis for these genes showed that there were four highly connected regions. The most relevant functions and pathways extracted from these subnetwork regions were involved in small G protein signaling pathways, oxidation–reduction in fatty acid metabolism and T cell proliferation.

Conclusion- Complicated network based pathways appear to play a role in the different pattern manifestations in patients with RA, and our results suggest that network-based pathways might be the scientific basis for TCM pattern classification.

Article:

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease affecting over 1% of the population.[1] The disease is characterized by synovial membrane hyperplasia, the progressive destruction of arthritic joints and inflammatory cell (including activated $CD4^+$ T cells) infiltration.[2] $CD4^+$ T cells play a crucial role in the pathogenesis of RA through multiple

mechanisms, including the following: the stimulation of the secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1; the induction of immunoglobulin production and matrix metalloproteinase secretion; and the stimulation of osteoclastogenesis.[3]

The clinical manifestations of RA are rather heterogeneous and are consequently difficult characterize biochemically.[4] Based on clinical phenotypes, Traditional Chinese Medicine (TCM) classifies RA patients as hot pattern, cold pattern or other.[5] The disease phenotype may be related to biological network effects consisting of hundreds to thousands of genes, whose expression levels change in various affected tissues and immune effector cells.[4]

Microarray technology is a massively parallel method for assessing gene expression changes at the genome scale. Many sets of disease-specific changes in gene expression levels are readily identified, providing information on individual disease mediators.[6] It has been demonstrated that the gene expression profiles in the T cells of patients with RA show evidence of multiple pathways of tissue destruction and repair.[7] Although TCM can classify RA into different patterns, little is known about the biological basis of the pattern classification in TCM. Fortunately, systems biology, which uses computational tools to predict biological networks arising from global, high-throughput data sets, can lead to a deeper understanding of this system.[8] In this study, based on gene expression profiles in $CD4^+$ cells of RA patients, we combined genome-wide expression analysis with methods of systems biology to identify functional networks for RA patients with hot and cold pattern and to identify connections between gene expression profiles and phenotype-based TCM pattern classifications.

METHODS

Patients and $CD4^+$ T-cell purification

Patients with RA (all females; age at diagnosis 42.8 ± 9.9 years old) were enrolled if they met the American College of Rheumatology criteria for RA for at least one year. Patients were classified as functional Class I, II, or III based on their current physical functioning level[9]; at the same time, they were categorized as having TCM cold or hot patterns. The TCM cold and hot patterns were determined according to the patient's symptoms. Patients with thirst, vexation, fever, and turbid yellow urine were categorized as TCM hot pattern, and patients with cold intolerance, cold feeling in the limbs and cold feeling in the joints were categorized as TCM cold pattern. Twenty-one RA patients with cold pattern and 12 RA patients with hot pattern were included in this study. Permission to conduct the study was granted by the local Ethical Committee for clinical research, and each patient was given informed consent prior to inclusion in the study. Peripheral blood (~15 ml) was collected in sodium-heparin containing vacuum tubes, and the $CD4^+$ T cell population was purified from the blood by negative selection using a $CD4^+$ T cell enrichment cocktail (StemCell, Canada).

Microarray and data analysis

Total RNA from $CD4^+$ T cells was extracted using TRIZol reagent according to the manufacturer's instructions. Probes were verified for amplification yield and incorporation efficiency by measuring the DNA concentration at 280 nm, Cy3 incorporation at 550 nm, and Cy5 incorporation at 650 nm. For each color, 10 pmol of incorporated dye was fragmented and resuspended in 500 μl of hybridization solution. Samples were hybridized to dual-color human

Whole Genome Microarrays (University of British Columbia, Canada) that contained four arrays of probes representing about 23,232 well-characterized transcripts. The arrays were hybridized in microarray hybridization chambers overnight at 42 °C. After washing, the slides were scanned with a GenePix 4000B scanner.

All data were analyzed using the SAS9.1.3 statistical package (Order no. 195557). The signal intensity of each expressed gene was globally normalized (LOWESS) using the R statistics program.[10] The ratio of cold pattern to hot pattern in RA patients at more or less than 1:2 was taken as the differential gene expression criteria. Statistical significance was tested using Student's *t*-test, with the statistical significance level set at $p < 0.05$. The candidate genes were adjusted by the Power Procedure of SAS software, which controls the False Discovery Rate (FDR). Genes were considered differentially expressed if the Power value was more than 0.85.

Protein–protein interaction network

Information on human protein–protein interactions was obtained from databases, including BIND (Biomolecular Interaction Network Database), BioGRID (The General Repository for Interaction Datasets), DIP (Database of Interacting Proteins), HPRD (Human Protein Reference Database), IntAct (Database system and analysis tools for protein interaction data) and MINT (Molecular Interactions Database). This information was complimented with curated relationships parsed from the literature using Agilent Literature Search.[11] These datasets are mostly based on experimental evidence. We did not include data that were deemed to be of lower quality. The protein–protein interaction network was visualized using Cytoscape software.[12]

Highly connected clusters of the integrated network

We integrated the database and the literature data mining networks and then used IPCA to analyze the characteristics of our network. The IPCA algorithm can detect densely connected regions in the interactome network.[13] Interactomes with a score greater than 2.0 and at least four nodes were taken as significant predictions in this study.

Gene ontology analysis

To identify the function of each cluster generated by IPCA individually, GO clustering analysis was performed with the proteins described in all subnetworks. For this purpose, the latest version of the Network Gene Ontology (BiNGO) tool[14] was used to statistically evaluate groups of proteins with respect to the existing annotations of the Gene Ontology Consortium. The degree of functional enrichment for a given cluster was quantitatively assessed (*p* value) by hypergeometric distribution, as implemented in the BiNGO tool. We selected the 10 GO biological categories with the smallest *p* values as significant.

RESULTS

Significantly differentially expressed genes between RA with TCM cold and hot pattern

To compare the gene expression in RA patients with TCM cold pattern and hot pattern, the Student's *t*-test was used to identify significantly differentially expressed genes ($p < 0.05$). Four genes were more highly expressed in RA patients with cold pattern than in hot pattern, and 21 genes had lower expression levels in cold pattern than in hot pattern (Table 1).

Table 1: Significantly differentially expressed genes between cold and hot pattern RA patients.

Gene ID	Symbol	Name	Cold/Hot ratio
NM_000811	GABRA6	Gamma-aminobutyric acid (GABA) A receptor, alpha 6	4.10
NM_001964	EGR1	Early growth response 1	2.17
NM_016582	SLC15A3	Peptide transporter 3	1.91
NM_006965	ZNF24	Zinc finger protein 24 (KOX 17)	1.49
NM_002241	KCNJ10	Potassium inwardly rectifying channel, subfamily J, member 10	0.70
NM_005345	HSPA1A	Heat shock 70kD protein 1A	0.68
NM_000234	LIG1	Ligase I, DNA, ATP-dependent	0.68
NM_032454	STK19	Serine/threonine kinase 19	0.67
NM_002105	H2AFX	H2A histone family, member X	0.66
NM_006111	MYO5B	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	0.66
AB046809	ZFYVE1	Zinc finger protein, subfamily 2A (FYVE domain containing), 1	0.66
NM_004992	MECP2	Methyl CpG binding protein 2 (Rett syndrome)	0.64
NM_014059	C13orf15	RGC32 protein	0.64
NM_014727	MLL4	KIAA0304 gene product	0.64
AB033075	ASAP1	Development and differentiation enhancing factor 1	0.63
NM_032936	TMEM60	DC32	0.62
AY013288	ASCC3	RNA helicase family	0.62
NM_000698	ALOX5	Arachidonate 5-lipoxygenase	0.61
NM_003827	NAPA	*N*-ethylmaleimide-sensitive factor attachment protein, alpha	0.58
NM_001382	DPAGT1	Dolichyl-phosphate (UDP-*N*-acetylglucosamine) *N*-acetylglucosaminephosphotransferase 1	0.57
NM_006598	SLC12A7	Solute carrier family 12 (potassium/chloride transporters), member 7	0.55
NM_054033	FKBP1B	FK506 binding protein 1B (12.6 kD)	0.55
NM_014585	SLC40A1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 3	0.55
U31099	PTGDR	Prostaglandin D2 receptor (DP)	0.53
U90902	TIAM1	Human clone 23612 mRNA sequence	0.50

Interaction networks for differentially expressed genes between RA patients with TCM cold and hot pattern

As shown in Fig. 1, the network containing 418 nodes and 4270 edges (the nodes represent proteins and the edges represent interactions between the proteins) was found to be related to the differential gene expression in RA patients with TCM cold and hot pattern.

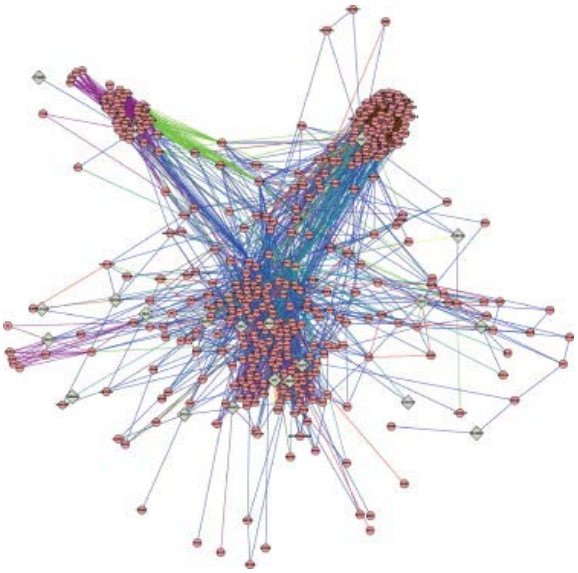

Figure 1: Protein–protein interaction network for cold and hot patterns. We used the official symbols of genes to search protein interaction information in protein interaction databases and the literature. Cytoscape, a network visualization tool, converts a list of genes (with or without accompanying expression information) into a relevant

network. Diamonds represent seed nodes. Cycles represent neighbor nodes. All edges represent interactions between the nodes.

Relevant functions and pathways extracted from highly connected clusters

In order to better understand the functions of the network shown in Fig. 1, four significantly highly connected clusters (a score of 2 was considered to be significant, which represented the log of the probability that the network was found by chance) were proposed by IPCA and visualized by Cytoscape (Fig. 2).

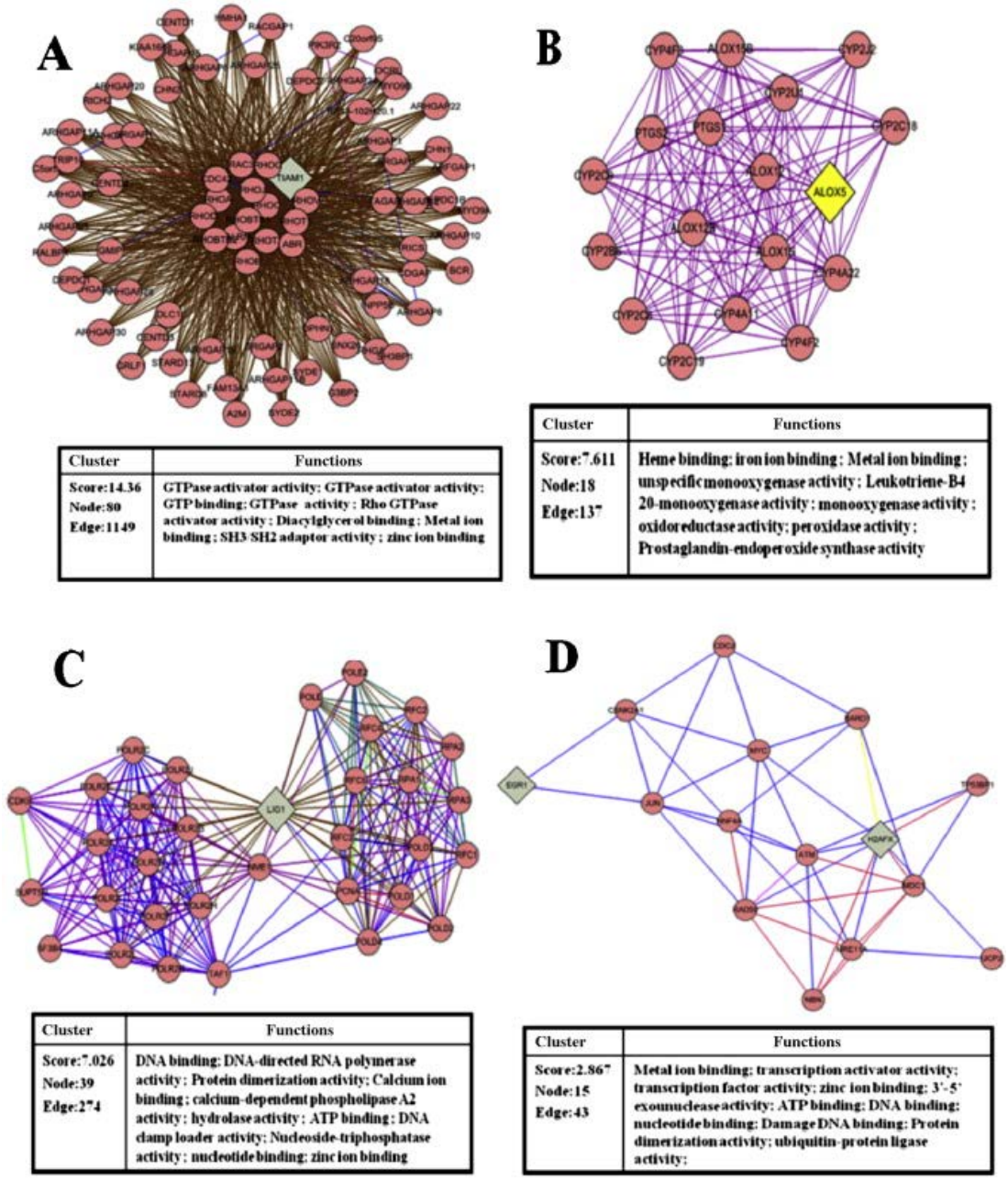

Figure 2: Subnetworks made up of highly connected regions and the functions of the nodes. Four significantly highly connected regions (or clusters) were proposed by IPCA, a clustering algorithm for analysis of the topological

structure of the protein interaction network, which allows for the detection of significant complexes or pathways. These subnetworks of highly connected regions were visualized by Cytoscape. Diamonds represent seed nodes. Cycles represent neighbor nodes. All edges represent interactions between the nodes. (A) cluster 1, (B) cluster 2, (C) cluster 3, (D) cluster 4.

The highest-scoring subnetwork (cluster 1 in Fig. 2A) was made up of 80 genes including 1 seed gene (a gene with significantly different expression levels in cold and hot pattern), which was the T-cell lymphoma invasion and metastasis 1 (TIAM1) gene. There were 15 genes in the center containing members of the ras homolog gene family, active BCR-related gene (ABR), and TIAM1; there were 65 genes in the circumference. Their functions include GTPase activation such as GTPase activator activity, GTP binding, and Rho GTPase activator activity. We used BiNGO tools to statistically evaluate groups of genes with respect to the present annotation categories of the Gene Ontology Consortium. The biological categories of this cluster were mostly associated with signal transduction (Table 2). This network may represent part of the small G protein signaling pathways. The expressed level of TIAM1 was higher in RA patients with TCM hot pattern than in cold pattern, implying that small G protein signaling pathways were activated in RA patients with hot pattern.

Table 2: Biological categories of the subnetworks.

Cluster	ID	Description	*p*-Value
Cluster 1	7165	Signal transduction	5.45E-48
	7154	Cell communication	1.04E-44
	51,244	Regulation of cellular process	1.99E-26
	50,791	Regulation of biological process	1.71E-25
	65,007	Biological process	1.09E-23
	7264	Small GTPase mediated signal transduction	2.39E-18
	7242	Intracellular signaling cascade	4.96E-18
	7266	Rho protein signal transduction	5.35E-14
	35,023	Regulation of Rho signal transduction	3.63E-11
	7265	Ras protein signal transduction	5.76E-11
Cluster 2	55,114	Oxidation reduction	3.62E-26
	6690	Icosanoid metabolic process	4.19E-22
	6631	Fatty acid metabolic process	6.99E-18
	32,787	Monocarboxylic acid metabolic process	2.78E-16
	43,449	Alkene metabolic process	2.85E-16
	6691	Leukotriene metabolic process	2.85E-16
	46,456	Icosanoid biosynthetic process	1.81E-15
	6633	Fatty acid biosynthetic process	5.57E-13
	19,752	Carboxylic acid metabolic process	1.15E-12
	6082	Organic acid metabolic process	1.24E-12
Cluster 3	6297	Nucleotide-excision repair, DNA gap filling	3.43E-42
	6289	Nucleotide-excision repair	2.83E-30
	6368	RNA elongation from RNA polymerase II promoter	8.73E-29
	6354	RNA elongation	2.60E-28
	6367	Transcription initiation from RNA poly merase	3.13E-24
	6352	Transcription initiation	5.38E-23
	43,284	Biopolymer biosynthetic process	2.25E-19
	6260	DNA replication	1.15E-18
	6139	Nucleobase, nucleoside, nucleotide	1.76E-18
	6366	Transcription from RNA polymerase II promoter	2.26E-18
Cluster 4	6974	Response to DNA damage stimulus	3.15E-10
	7049	Cell cycle	1.81E-09
	6281	DNA repair	3.71E-09
	22,402	Cell cycle process	3.74E-09
	7126	Meiosis	6.98E-09
	51,327	M phase of meiotic cell cycle	6.98E-09
	51,321	Meiotic cell cycle	8.05E-09
	6950	Response to stress	4.59E-08
	6302	Double-strand break repair	4.76E-08

The second subnetwork (cluster 2 in Fig. 2B) was made up of 18 nodes, including prostaglandin-endoperoxide synthase, members of the cytochrome P450 superfamily of enzymes and the lipoxygenase gene family. The functions of these genes include heme binding, iron ion binding, metal ion binding, unspecific monooxygenase activity, leukotriene-B4 20-monooxygenase activity, oxidoreductase activity, peroxidase activity and prostaglandin-endoperoxide synthase activity. Genes in cluster 2 were associated with oxidation–reduction reactions, eicosanoid metabolic processes and fatty acid metabolic processes (Table 2). Based on this information, it was deduced that the most relevant function of this subnetwork was related to oxidation–reduction reactions in fatty acid metabolism. The expressed level of the seed gene arachidonate 5-lipoxygenase (ALOX5) in RA patients with TCM hot pattern was higher than that in cold pattern (Table 1). These imply that fatty acid metabolism might be increased in RA patients with TCM hot pattern.

The third subnetwork (cluster 3 in Fig. 2C) contains 39 nodes, which can be divided into two groups jointed by DNA ligase 1 (LIG1) and NDP kinase A (NME1). The first group includes subunits of RNA polymerase II, subunits of the splicing factor 3B, TATA box binding protein (TBP)-associated factor (TAF1), the splicing factor 3b subunit 4 (SF3B4), cyclin-dependent kinase 9 (CDK9), and the suppressor of Ty 5 homolog (SUPT5H). Mutations in LIG1 that lead to DNA ligase I deficiency result in immunodeficiency and increased sensitivity to DNA-damaging agents (http://www.ncbi.nlm.nih.gov/gene/3978). The second group is the DNA polymerase delta complex. The functions of these genes (Fig. 2C) and the biological categories of the cluster 3 (Table 2) were associated with RNA transcription and DNA replication.

The fourth subnetwork (cluster 4 in Fig. 2D) contains the jun proto-oncogene (JUN), the v-myc myelocytomatosis viral oncogene homolog (MYC), and the early growth response 1 (EGR1) gene (Fig. 2D). Genes in cluster 4 were associated with response to DNA damage stimulus and the cell cycle (Table 2). Therefore, both the cluster 3 and 4 genes appeared to be involved in cell proliferation. EGR1 suppresses mitogenesis, whereas the H2A histone family member X (H2AFX) and LIG1 promote the cell cycle. The expressed level of the seed gene EGR1 was lower in RA patients with TCM hot pattern than in RA cold pattern whereas H2AFX and LIG1 were in opposite (Table 1). This implies that T cell proliferation increases in RA patients with hot pattern compared to RA patients with cold pattern.

DISCUSSION

In this study, we used the microarray analysis of gene expression in $CD4^+$ T cells to outline network-based biomarkers for the classification of RA patients with TCM cold and hot pattern. We observed an increased expression of genes related to small G protein signaling pathways, fatty acid metabolism and T cell proliferation in RA patients with hot pattern.

Microarray techniques are widely used to gain insight into the molecular complexity of disease.[15] The identification of gene–gene interactions rather than independently altered genes can provide a better understanding of the underlying molecular mechanisms of TCM pattern expression in patients with RA. In our study, network-based pathways or clusters were obtained from RA patients with cold and hot pattern by analyzing differentially expressed genes in $CD4^+$ cells taken from RA patients. Four subnetworks were found that can be used to classify RA patients into cold and hot pattern phenotypes.

RA is a rather heterogeneous disease, and the response to biomedical therapies is inconsistent. This suggests that RA patients could be divided into different subsets in order to select of different therapies. We have shown in a previous study that cold pattern RA patients responded better to biomedical therapy than hot pattern RA patients.[16] However, it is difficult to identify a single biomarker which can accurately classify RA patients into cold and hot pattern types. Thus, there are three major limitations in this paper. First, limitations of the protein–protein interaction network analysis might miss significant findings regarding the most useful signatures for TCM pattern classification.[17] In this study, we combined literature data mining with real data and an evidence-based dataset in order to accurately assess the real signatures of TCM patterns. We also combined other analysis approaches, such as the use of IPA software. This technique could permit comparisons not only between cold and hot pattern RA patients, but also between cold and hot pattern RA patients and healthy controls. Second, the sample size might have caused us to miss significant findings. Although we tested 33 patients with RA and combined this analysis with other data from the public, we need to conduct further studies in other groups, including RA patients with other types of TCM patterns (such as the deficiency pattern). Further studies are needed to verify the biological networks that may be the basis for cold and hot pattern classifications in patients with RA. Finally, we only identified the networks related to hot pattern RA patients because the only comparisons made were between hot and cold pattern patients. Further comparisons between cold and hot pattern RA patients and healthy controls are needed.

This study found that RA patients with hot pattern had an increased expression of genes related to small G protein signaling pathways, fatty acid metabolism and T cell proliferation. Previous studies [18] and [19] have shown that immune factors predominate in the hot pattern network. This is partly supported by our results, which show that small G protein signaling pathways were activated and that the expressed level of TIAM1 and ALOX5 may be higher in RA hot pattern than in RA cold pattern. TNF-α plays an important role in RA by activating T cells through small G protein signaling pathways.[20] Cell polarization is required for T cell processes such as activation, proliferation and invasion in response to inflammatory factors. [21] and [22] Intracellular localization and activation of the partitioning defective polarity complex are initiated by RAP1 and require CDC42 activity.[23] The RAC activator TIAM1 associates with both RAP1 and components of the partitioning defective complex. This pathway may function to connect the partitioning defective polarity complex to RAP1 and to regulate the RAC-mediated actin remodeling required for T cell polarization.[24] Consistent with these findings, TIAM1-deficient T cells are impaired in RAP1- and chemokine-induced polarization and chemotaxis.[25] These indicate that T cell proliferation, invasion and secretion of proinflammatory cytokines might be increased in hot pattern RA patients compared to cold pattern RA patients.

In RA, activated T cells secrete proinflammatory cytokines and release lipid inflammatory mediators, such as prostaglandin E2 and leukotriene B4. These substances are derived from arachidonic acid in fatty acid metabolic processes and induce fever, increase vascular permeability and vasodilatation, enhance local blood flow, cause pain, induce the release of lysosomal enzymes, induce the release of reactive oxygen species by granulocytes and increase the production of IL-6. The combined effect is to aggravate inflammation.[26] ALOX5, a member of the lipoxygenase gene family, plays a dual role in the synthesis of leukotrienes from arachidonic acid.[27] This protein catalyzes the conversion of arachidonic acid to 5(S)-

hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid and further to the allylic epoxide 5(S)-trans-7,9-trans-11,14-cis-eicosatetrenoic acid (leukotriene A4). Our results suggest that lipid inflammatory mediators are increased in RA hot pattern patients, although they were not included in the networks related to the general hot pattern.

This study showed that the activation and invasion of T cells were increased by small G protein signaling pathways and that release of lipid inflammatory mediators increased due to augmentation of fatty acid metabolism in RA patients with hot pattern. This has been partly supported by a publication demonstrating that RA patient profiles fall into two molecularly distinct subsets based on hierarchical clustering of gene-expression data. One subset had a profile characterized by infiltration of leukocytes and overexpression of adaptive immune response genes in T cells.[7] Compared to the results of our previous study,[28] more new genes (such as those involved in lipid metabolism and G protein signaling pathways) were found to be related to RA hot pattern. However, some related networks, such as those related to immune regulation and cell proliferation, were found in both datasets. This might be due to an incompatibility of two independent gene expression profiles caused by the natural instability of the microarray assay or to an improved microarray assay.

Cold and hot patterns are two key terms in TCM theory, and these patterns can be found in many diseases.[29] Additional efforts have been made to investigate the biomedical basis for the TCM cold and hot pattern classifications. By using the methods of literature mining, network analysis and topological comparison, Li[18] found that hormones predominate in the cold pattern network, immune factors predominate in the hot pattern network, and that these two networks are connected by neurotransmitters. Moreover, Ma[19] claimed that cold pattern-related genes play an essential role in energy metabolism and are tightly correlated with genes regulating neurotransmitters, hormones and cytokines in the neuro-endocrine-immune (NEI) interaction network. Yang[30] suggested that the cold pattern might be caused by physiological imbalances and/or disordered metabolic processes. These results all show a biological basis for the cold and hot patterns in TCM. However, using microarray data, we demonstrated the presence of different biological networks in patients with the same specific disease (RA) but different cold and hot pattern classifications. Similarly, differences in the general networks, including immune factors and cytokines, were also shown in our network analysis.

In conclusion, small G protein signaling pathways, oxidation–reduction reactions in fatty acid metabolic processes and T cell proliferation appear to be involved in manifestations of cold and hot patterns in RA patients. The results suggest that network-based pathways may be a scientific basis for TCM pattern classifications.

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