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Functional and Molecular Characterization for the Damp-Obstructed Rat Model in Chinese Medicine

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Abstract: Functional and molecular characterization was performed on the major organs of damp-obstructed rats by applying expression datasets of microarray experiments and real-time RT-PCR. Gene ontology repertoires, i.e. cellular component, molecular function, and biological process were used to classify differentially expressed genes in the major organs of rats upon treatment of dampness. As to the cellular component, over-expression of genes associated with the plasma membrane was observed in the stomach, spleen, kidney, heart, liver, and lung. Genes associated with translational machinery, endoplasmic recticulum membrane, Golgi apparatus, and nuclear envelope were down-regulated in the stomach. Concerning the molecular function, genes associated with oxidoreductase activity were up-regulated in the stomach, spleen, kidney, lung, and brain. Channel activity, membrane receptor, and electron transporter activity were up-regulated in stomach, kidney, and lung. Regarding the biological process, genes associated with signal transduction were up-regulated in the stomach, while genes associated with biosynthesis and ATP metabolism were down-regulated. In the spleen, melanin biosynthesis was up-regulated while hormone-related activities were down- regulated. In the kidney, genes associated with nucleotide biosynthesis and ATP metabolism were depressed. In the heart and liver, apoptosis was up-regulated while immune response and RAS signal transduction were down-regulated. Interestingly, genes associated with oncogenesis were up-regulated in the stomach and kidney. Functional fingerprints indicated that dampness weakened membrane structures, depressed metabolic activity (especially ATP metabolism), damaged matrix proteins, enhanced signal transduction, and revealed a positive association with oncogenesis. To quantify the functional impact at the molecular level, mRNA levels of key genes were determined by real-time RT-PCR. The results indicated that ATP storage in kidney, spleen, and stomach was depleted in damp-obstructed rats. We propose that oxidative stress, membrane integrity, melanin biosynthesis, ion channel activity, and ATP metabolism might

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be hallmarks for damp-obstructed rats. Our results also suggested dampness as a pathogenic factor in rats which is possibly associated with enhanced liabilities of cancer.

Keywords: Damp Obstruction; Gene Ontology; Real-Time RT-PCR; Functional Categories.

Introduction

Damp obstruction refers to the stagnation of vital energy (qi) caused by dampness resulting in dysfunctional body and limb movement, as well as the impairment of spleen and stomach digestive function (Li and Yen, 2000). Dampness (humidity) is one of the major pathogenic factors on the human body as defined by Chinese medicine. Dampness is characterized by fluidity, latency, universality, and persistence. Dampness is sticky, retards flow of qi, and consequently causes disregulation of the "middle-energizer" (Huang *et al*., 2000). In Chinese medicine, the general term for this humidity-induced syndrome is "damp obstruction" as described in the *Yellow Emperor Classic of Internal Medicine* (*Huang Di Nei Jing*). In general, damp obstruction is defined as a moisture-caused imbalance of organs. Because the spleen and stomach belong to "tu" (earth) among the five elements, they are the first organs affected. Damp obstruction presents a clear case for the flow of vital energy and obstruction/subjugation of the five elements; therefore, it is hereby singled out as an ideal system to approach with modern molecular biology.

We have previously performed microarray analyses on the damp-obstructed rat model (Huang and Hong, 2005). Microarray simultaneously presents the expression of tens of thousands of genes on a genomic scale (Schena *et al*., 1995; Alizadeh *et al*., 2000). Differentially expressed genes and expression profiles specific to damp-obstructed organs can be obtained. However, statistical treatment that acquires functional and molecular themes for dampness is yet to be explored.

The current study is based on the functional clustering analyzing program EASE, developed for data mining microarray informatics (Hawse *et al*., 2003; Hosack *et al*., 2003). Gene ontology annotation information derived from LocusLink database was applied, sorted, and calculated with the EASE program. Statistical significance will be discussed.

Methods

Damp-Obstructed Rat Model

Damp-obstructed rats were established based on previous reports with minor modification (Huang *et al.*, 2000; Kuo and Yan, 1988; Lu, 1995; Lu and Ma, 1994). Male Sprague-Dawley rats, weighing 150–200 g, were obtained from the National Science Council Animal Center. All procedures were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals. The rats were randomly housed in groups of two per wire-mesh cage (39 × 26 × 21 cm) for at least 1 week. Animals were divided into two groups: damp-obstructed group and control group. Each group contained six rats.

For control group, rats were placed in a controlled environment of 23 ± 1ºC and 50% relative humidity with free access to standard food in pellets (supplied and designed by Fusow Industry Co. Ltd., Taiwan) and tap water, on a 12-hour light/dark cycle. For damp-obstructed group, rats were placed in a controlled environment of 31 ± 1°C and 94 ± 5% relative humidity with free access to standard food in pellets and tap water, on a 12-hour light/dark cycle. In addition to normal chow, damp-obstructed rats were given 18 ml 20% bee honey and 5 g lard daily. The control group was handled with the identical procedure but received the same volume of normal saline instead.

Serum Biochemical Assays

Animals were fasted for 8 hours prior to blood sampling. Anesthetization was performed by intraperitoneal injection of pentobarbital (45 mg/kg, ip). Five hundred microliters of blood were drawn from the tail vein of each animal. Serum in plain vial was separated at 4°C in a cooling centrifuge for 15 minutes at 2000×*g*. Serum glutamate pyruvate transaminase activity (GPT), glutamate oxaloacetate transaminase activity (GOT), low-density lipoproteins (LDL), high-density lipoproteins (HDL), total cholesterol, triglyceride (TG), alkaline phosphatase activity (ALP), lactate dehydrogenase activity (LDH), serum creatine (CREA), serum creatine phosphokinase activity (CPK), and blood urea nitrogen (BUN) were determined using Roche COBAS MIRA automatic assay machine using standard reagents purchased from Roche Co.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT^{2}PCR)

Total RNA was extracted using the protocol supplied with the TRI-reagent (Molecular Research Center, Inc., USA). Quality of RNA was examined by agarose gel electrophoresis and also by OD 260/280 ratio (greater than 2.0). Total cellular RNA (2.5 μg per 20 μl reaction) was reverse transcribed using polydT(15) and Superscript RT II (Invitrogen, USA) according to the manufacturer specified conditions. A control without reverse transcriptase was also generated for each RNA sample.

Real-time PCR analysis was performed using iQ™ SYBR green supermix (Bio-Rad) according to manufacturer's instructions with the specific primers for selected genes and primers for 18L ribosomal RNA as reference RNA. Cycle time (Ct) was measured using the iCycler™ and its associated software (Bio-Rad) (Morrison *et al*., 1998). Relative transcript quantities were calculated by the ΔΔCt method using 18L ribosomal RNA as a reference amplified from samples. Normalized samples were then expressed relative to the average ΔCt value for untreated controls to obtain relative fold-change in expression levels. Fold change in mRNA expression was expressed as $2^{\Delta\Delta Ct}$. ΔCt is the difference in threshold cycles for the sample mRNAs and 18L rRNA. ΔΔCt is the difference between ΔCt control and ΔCt damp-obstructed sample. Values for fold-induction varied less than 5% among replicates.

One-factor ANOVA was used to analyze differences in mRNA levels as detected by real-time PCR between the treatment and the control groups.

Gene Ontology and EASE

Differentially expressed genes were obtained from previous microarray datasets (Huang and Hong, 2005). Genes with expression folds beyond standard deviation were considered up- or down-regulated. The LocusLink identifiers associated with genes on the microarray were updated using database provided by SWISS-PROT. A total of 8262 genes out of 9600 genes on the microarray were associated with a unique LocusLink number and served as the basis for further statistical analysis. Lists of identifiers containing differentially expressed genes were generated. For damp-obstructed stomach, the list contains 734 up-regulated and 760 down-regulated genes. For damp-obstructed spleen, the list contains 1099 up-regulated and 1106 down-regulated genes. For damp-obstructed kidney, the list contains 717 up-regulated and 777 down-regulated genes. For damp-obstructed heart, the list contains 845 up-regulated and 920 down-regulated genes. For damp-obstructed liver, the list contains 601 up-regulated and 428 down-regulated genes. For damp-obstructed lung, the list contains 690 up-regulated and 751 down-regulated genes. For damp-obstructed brain, the list contains 641 up-regulated and 456 down-regulated genes.

The EASE program was downloaded from DAVID web page (Database for Annotation, Visualization, and Integrated Discovery, http://apps1.niaid.nih.gov/david/upload.asp). Functional principles, i.e. cellular component, molecular function, and biological function are provided from LocusLink database and incorporated into the EASE program. The EASE program counts listed hits, compares the expected hits in each functional category, and calculates Fisher exact probabilities (Hawse *et al*., 2003; Hosack *et al*., 2003). The Fisher exact probability for over-representation was calculated using Gaussian hypergeometric probability distribution that describes sampling without replacement from a finite population consisting of two types of elements. The EASE score, the upper bound of Jackknife Fisher exact probabilities, was obtained to rank categories significantly up- or down-regulated among all populations. The EASE score is a conservative adjustment of the Fisher exact probability that strongly penalizes categories supported by few genes and negligibly penalizes categories supported by many genes.

Results

Classification of Functional Gene Clusters Differentially Expressed in Damp-Obstructed Rats

The three organizing principles of gene ontology are molecular function, biological process, and cellular component. A gene product might be clustered to one or more molecular functions and to one or more biological processes; it might be associated with one or more cellular components.

Dampness could be better characterized by clusters of functionally related genes differentially expressed between damp-obstructed and control rats. The number of genes corresponding to each category among the differentially expressed genes was counted and compared with the number of genes expected for each category. Significant differences were represented as the EASE scores; the upper bound of the distribution of Jackknife

Fisher exact, were calculated. A false discovery rate was also calculated based upon the number of categories having at least one gene in the list of differentially expressed genes (Cleveland, 1979). All functional categories with an EASE score of less than 0.05 and their number of corresponding genes were identified in our differentially expressed gene lists. The functional categories demonstrating significant variations among differentially expressed genes appear as various subsets for different organs (Tables 1 to 3).

Table 1. Functional Categories of Cellular Components Differentially Expressed in Damp-Obstructed Rats

Organ	Up-Regulation	Down-Regulation
Stomach	Tight junction Plasma membrane Intercellular junction Cell junction	Endomembrane system Eukaryotic 48S initiation complex Intracellular components Cytoplasm Eukaryotic 43S pre-initiation complex Endoplasmic reticulum Small ribosomal subunit Endoplasmic reticulum membrane Nuclear envelope-endoplasmic reticulum network Eukaryotic translation initiation factor 4 complex Collagen Proton-transporting two-sector ATPase complex Ribonucleoprotein complex Golgi membrane Basement membrane Golgi apparatus Mitochondrial electron transport chain Hydrogen-translocating F-type ATPase complex Proton-transporting ATP synthase complex Sodium/potassium-exchanging ATPase complex
Spleen	Spliceosome complex Mediator complex Plasma membrane	Plasma membrane
Kidney		Polysome Proton-transporting two-sector ATPase complex
Heart	Integral to plasma membrane Plasma membrane Integral to membrane Actin cytoskeleton Membrane	Cell-matrix junction
Lung	Integral to plasma membrane Plasma membrane Cell fraction Integral to membrane	Intracellular components Nuclear membrane Cytoskeleton Chloroplast
	Membrane fraction	Pore complex Nuclear pore Cytoplasm Actin cytoskeleton Nucleus
Brain	Extracellular Extracellular space	Cell Cell cortex

Table 2. Functional Categories of Molecular Functions Differentially Expressed in Damp-Obstructed Rats

Organ	Up-Regulation	Down-Regulation
Stomach	Oxidoreductase activities* Protease inhibitor activities Kinase activity Copper ion binding Phosphotransferase activity, alcohol group as acceptor electron transporter activity	RNA binding Electron transporter activity Hydrogen ion transporter activity Monovalent inorganic cation transporter activity Ubiquinol-cytochrome-c reductase activity Oxidoreductase activities* Extracellular matrix structural constituent Benzodiazepine receptor activity Peptidyl-prolyl cis-trans isomerase activity Histone deacetylase activity Deacetylase activity Cis-trans isomerase activity
Spleen	Oxidoreductase activities	Oxidoreductase activities Monooxygenase activity RNA polymerase II transcription factor activity, enhancer binding
Kidney	Heme-copper terminal oxidase activity Cytochrome-c oxidase activity Oxidoreductase activities Transporter activity Protein binding Actin bundling activity 3′,5′-cyclic-nucleotide phosphodiesterase activity Excitatory extracellular ligand-gated ion channel activity Electron transporter activity Protein transporter activity	Cell adhesion molecule activity Receptor signaling complex scaffold activity Hematopoietin/interferon-class (D200-domain) cytokine receptor activity
Heart	Transferase activity, transferring sulfur-containing groups Glucosyltransferase activity Solute:cation symporter activity Sulfotransferase activity	ATP-dependent helicase activity MHC class II receptor activity Helicase activity Nucleic acid binding
Heart	Solute:sodium symporter activity	DNA binding RNA binding Single-stranded DNA binding DNA helicase activity Single-stranded RNA binding Sodium channel activity Class II major histocompatibility complex antigen mRNA binding

Table 2. (*Continued*)

Organ	Up-Regulation	Down-Regulation
Liver	Enzyme binding	Protein binding Monocarboxylic acid transporter activity Transferase activity, transferring acyl groups Transcriptional repressor activity Protein self-binding
Lung	Potassium channel activity Glutamate-gated ion channel activity Inotropic glutamate receptor activity Monocarboxylate channel activity Glutamate channel activity Kainate selective glutamate receptor activity Ion channel activity Voltage-gated ion channel activity Alpha-type channel activity Transmembrane receptor activity Channel pore class transporter activity Excitatory extracellular ligand-gated ion channel activity Oxidoreductase activities Cell adhesion molecule activity Transcription factor activity	Cytoskeletal protein binding Actin binding Phosphatidylinositol-4,5-bisphosphate 3-kinase activity MHC class II receptor activity RNA binding Pyrophosphatase activity
Lung	Protein kinase regulator activity Kinase regulator activity Oxidoreductase activities Blood coagulation factor activi	Molecular function unknown Toxin activity C-C chemokine receptor activity C-C chemokine binding

*Oxidoreductase activities in up- and down-regulated categories of stomach contain different subsets of categories.

Table 3. Functional Categories of Biological Processes Differentially Expressed in Damp-Obstructed Rats

Organ	Up-Regulation	Down-Regulation
Stomach	Cell communication Phosphorylation Oncogenesis Cytoskeleton organization and biogenesis Response to external stimulus Intracellular signaling cascade Protein amino acid phosphorylation Response to abiotic stimulus Cell organization and biogenesis Protein modification Signal transduction	Energy pathways Macromolecule biosynthesis Electron transport ATP metabolism Acetate biosynthesis Acetate metabolism Acetyl-CoA biosynthesis Biosynthesis Ribonucleoside triphosphate metabolism Purine nucleoside triphosphate metabolism Purine ribonucleoside triphosphate metabolism Main pathways of carbohydrate metabolism Protein metabolism Vesicle-mediated transport
Spleen	Tyrosine metabolism Vitamin cofactor transport Enzyme-linked receptor protein signaling pathway Response to external stimulus Melanin biosynthesis Melanin biosynthesis from tyrosine Cell surface receptor-linked signal transduction vision Aromatic amino acid family metabolism Transmembrane receptor protein tyrosine kinase Signaling pathway	Central nervous system development Hormone biosynthesis nucleoside metabolism C21-steroid hormone biosynthesis C21-steroid hormone metabolism Transmembrane receptor protein tyrosine Kinase signaling pathway Enzyme-linked receptor protein signaling pathway Hormone metabolism
Kidney	Regulation of transcription from Pol II promoter Secretory pathway Protein transport Intracellular protein transport Electron transport Oncogenesis Protein complex assembly Development Transcription from Pol II promoter	Cell communication Ribonucleoside triphosphate biosynthesis Purine nucleoside triphosphate biosynthesis Purine ribonucleoside triphosphate biosynthesis Nucleoside triphosphate biosynthesis Purine nucleoside triphosphate metabolism Ribonucleoside triphosphate metabolism Purine ribonucleoside triphosphate metabolism ATP biosynthesis Nucleoside phosphate metabolism Cell adhesion Nucleoside triphosphate metabolism Cellular process mRNA cleavage Response to bacteria ATP metabolism Signal transduction Regulation of neurotransmitter levels

Table 3. (*Continued*)

Organ	Up-Regulation	Down-Regulation
Heart	Carbohydrate biosynthesis Carbohydrate metabolism Anion transport Mitotic anaphase Antimicrobial humoral response (sensu Vertebrata) Antimicrobial humoral response Positive regulation of programmed cell death Induction of apoptosis Positive regulation of apoptosis Regulation of programmed cell death Induction of programmed cell death Humoral defense mechanism (sensu Vertebrata) Receptor-mediated endocytosis	RAS protein signal transduction Antigen processing Antigen presentation Physiological process Response to biotic stimulus Antigen presentation, exogenous antigen Antigen processing, exogenous antigen via MHC class II Non-covalent chromatin modification Chromatin remodeling Defense response Response to stress Response to pest/pathogen/parasite Embryonic development Immune response Intracellular signaling cascade Vesicle-mediated transport Complement activation Activation of MAPK Regulation of pH mRNA processing
Liver	Transcription from Pol II promoter Regulation of growth rate Positive regulation of growth rate Regulation of apoptosis Positive regulation of growth Intracellular transport Growth Response to metal ion Perception of sound	Response to biotic stimulus Immune response Defense response Response to external stimulus Response to pest/pathogen/parasite Response to stress Innate immune response Inflammatory response Response to wounding Humoral immune response Humoral defense mechanism (sensu Vertebrata) Circulation Antimicrobial humoral response (sensu Vertebrata) Antimicrobial humoral response Cell motility

Table 3. (***Continued***)

Organ	Up-Regulation	Down-Regulation
Lung	DNA replication	
	S phase of mitotic cell cycle	
	DNA replication and chromosome cycle	
	Potassium ion transport	
	Cellular process	Actin cytoskeleton organization and biogenesis
	Central nervous system development	Actin filament-based process
	Cell proliferation	Antigen presentation
	Cell cycle	Antigen processing
	Pregnancy	Regulation of Wnt receptor signaling pathway
	Transcription from Pol II promoter	Regulation of transcription from Pol II promoter
	Response to external stimulus	Peptidyl-amino acid modification
	Homeostasis	
	Cell growth and/or maintenance	
	Protein complex assembly	
	Metal ion transport	
Brain	Response to abiotic stimulus	G-protein signaling, coupled to IP3 second Messenger (phospholipase C activating)
	Response to external stimulus	
	Perception of abiotic stimulus	
	Perception of external stimulus	
	Vision	
	Chromosome segregation	
	Perception of light	
	Response to radiation	
	Sensory perception	
	Response to light	
	Taxis	
	Chemotaxis	
	Phosphate metabolism	
	Phosphorus metabolism	
	Aromatic amino acid family catabolism	
	Response to chemical substance	

Cellular Components

A cellular component is a component of a cell represented by an anatomical structure or a gene product group. Thus, the cellular component might reveal structural information and locations of a pathogenic attack.

In the stomach, over-expressed genes were associated with integral components of plasma membrane or junction proteins, while down-regulated expression was associated with inner membrane systems, i.e. ER, Golgi apparatus, basement membrane, and nuclear pore complex (Table 1). We also noticed that genes associated with transcriptional activity, i.e. ribosomal subunit and transcriptional factors, and transporter proteins were down-regulated. In the spleen, genes associated with plasma membrane were significantly disturbed. In the heart, genes clustered to plasma membrane and cytoskeleton were up-regulated, while cell-matrix and junction proteins were down-regulated. In the lung, genes associated with plasma membrane were up-regulated, while cytoskeleton and nuclear pore proteins were down-regulated. In the brain, only genes associated with extracellular function were up-regulated. Interestingly, cortex function was down-regulated in brain.

In general, plasma membrane was enhanced, while cytoskeletal and nuclear pore proteins were down-regulated.

Molecular Function

Molecular function describes activities of the gene product at the molecular level. The clustering of molecular function might reveal functional alternation in a detailed description.

In the stomach, stimulation and repression of oxidoreductase activities and electron transporter activities were observed. Protease inhibitor activities were enhanced, while deacetylase activities were down-regulated (Table 2). In the spleen, oxidoreductase activities were either enhanced or depressed in different subcategories. In kidney, oxidoreductase activities, transporter activities, and channel activities were up-regulated, while cell adhesion and receptor activities were down-regulated. In the heart, transferase activities and symporter activities were elevated, while nucleotide binding activities, channel activities, and immune response were down-regulated. In the lung, oxidoreductase activities, cell adhesion, channel, and receptor activities were increased, while cytoskeleton and immune response were decreased. In the brain, oxidoreductase activities, kinase, and blood coagulation were enhanced, while chemokine activities were reduced.

Overall expression of oxidoreductase activities was disturbed. Activities associated with membrane, such as electron transporter activity, channel, and receptor, were up- or down-regulated.

Biological Process

A biological process is generally composed of one or more distinct but ordered steps of molecular function. The process usually accomplishes a specific function for the

cell, e.g. cell growth and maintenance, signal transduction, or pyrimidine metabolism. Biological processes assemble molecular functions and point to trends of functional differences for cells.

In the stomach, functions associated with oncogenesis, signal transduction, and cytoskeleton biosynthesis were up-regulated, while functions for biosynthesis and ATP metabolism were down-regulated (Table 3). In the spleen, tyrosine metabolism and related pathways, especially melanin biosynthesis and metabolism, were up-regulated, while signaling pathway and hormone metabolism were depressed. In the kidney, oncogenesis, electron transporter, and protein biosynthesis were enhanced, while ATP-related metabolic pathways and signal transduction were repressed. In the heart, apoptosis, anion transporter, and carbohydrate-related pathways were enhanced, while immune response, stress response, and RAS-associated signal transduction were repressed. In the liver, apoptosis and cell growth were enhanced, while immune response, inflammatory response, stress response, and cell mobility were depressed. In the lung, cell proliferation, cell growth, and potassium transporter were enhanced, while cytoskeleton and immune response were down-regulated. In the brain, cellular response, chemotaxis, and phosphate metabolism were up-regulated, while G-protein signaling pathway was down-regulated.

Overall, oncogenesis, cell response, electron transporter, and cell growth were enhanced, while ATP metabolism, hormone activity, immune response, inflammatory response, and cytoskeleton were depressed.

Quantitative Analysis for Specific Gene Expression

To validate the expression dataset of microarray experiments and further confirm the differential expression of key genes indicated from functional analysis, real-time reverse transcription polymerase chain reaction (RT^2PCR) was performed with selected genes (Table 4, Fig. 1) of the kidney, spleen, stomach, lung, heart, and liver of damp-obstructed rats and normal controls (Morrison *et al*., 1998). Results of RT^2PCR were shown as $\Delta\Delta Ct$ which are comparable to the logarithmic fold change of microarray expression data (Table 5). The results indicated that $\Delta\Delta Ct$'s from RT^2PCR of damp-obstructed rats versus normal were consistent with expression folds derived from microarray datasets.

Differential expression of Atp5g1, Atp6l, Cldn7, Grin2d, Kif11, PPP1R12A, Pea15, Kcna4, Prm2, Tk1, and Tubb was disclosed for most damp-obstructed tissues. The differential mRNA levels indicated destructive consequences of dampness on animals.

Discussion

Statistical analysis applying the EASE program to classify differentially expressed genes in each major organ of damp-obstructed rats was performed. Functional clusters were mapped to gene ontology repertoires provided in LocusLink database. Cellular component, molecular function, and biological process were used to classify genes differentially expressed in damp-obstructed rats.

Table 4. Genes Selected for RT2PCR

Symbol	Gene Name	Gene Locus*
Atp5g1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1	NM_017311
Atp6l	ATPase, H+ transporting, lysosomal (vacuolar proton pump) 9 kD	NM_130823
Cldn7	Claudin 7	XM_213349
Grin2d	Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	NM_022797
Kif11	Kinesin-like 1	AF035955
PPP1R12A	Myosin phosphatase, target subunit 1	NM_053890
Pea15	Phosphoprotein enriched in astrocytes 15	XM_213942
Kcna4	Potassium voltage-gated channel, shaker-related subfamily, member 4	NM_012971
Prm2	Ribonucleotide reductase M2 polypeptide	XM_216671
Tk1	Thymidine kinase 1, soluble	XM_236470
Tubb	Tubulin, beta polypeptide	XM_214461

*Gene locus indicates mRNA sequence deposited in GenBank.

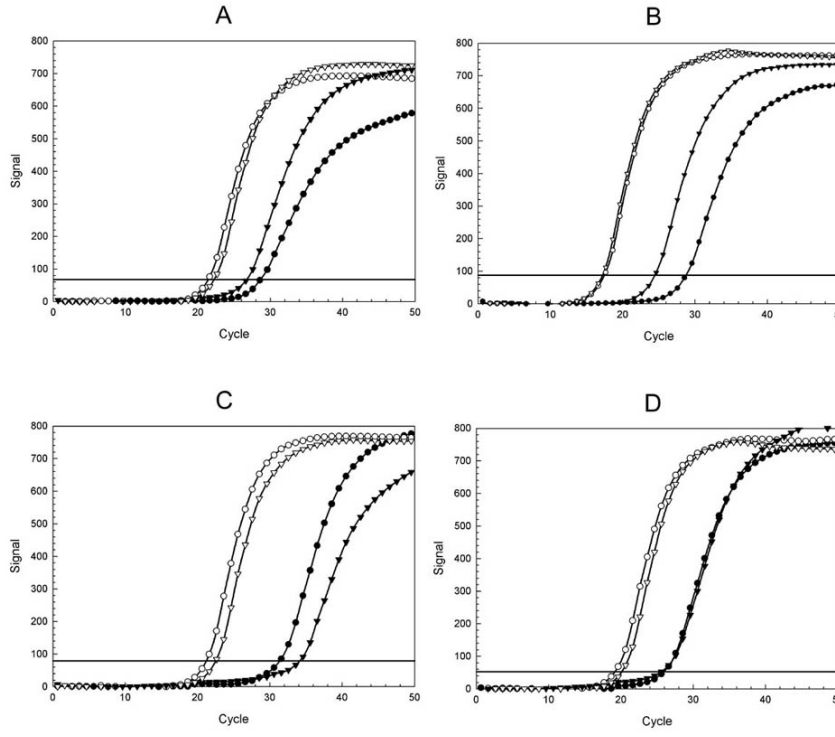

Figure 1. RT-PCR analyses of ATP6l in damp-obstructed rat organs versus control. RT^{2}PCR was performed using primers against ATPase (Atp6l) and 18L rRNA for the kidney, spleen, stomach, and lung from damp-obstructed rats versus normal control. Four typical results shown are kidney (A), spleen (B), stomach (C), and lung (D). Each figure contained real-time measurements for Atp6l in damp-obstructed rat (filled triangle), Atp6l in control (filled circle), 18L in damp-obstructed rat (open triangle), and 18L in control (open circle). Threshold was calculated and was shown as a horizontal line. This dataset shows that Atp6l was up-regulated in kidney, spleen, lung, and down-regulated in stomach. Detailed results are shown in Table 5.

Table 5. Comparison of RT2PCR and Microarray Expression for Selected Genes in Damp-Obstructed Rats versus Normal Rats

Gene Symbol	Kidney		Spleen		Stomach		Lung		Heart		Liver		Brain	SD[†]
	Δ ct[*]	M[†]	Δ ct	M	Δ ct	M	Δ ct	M	Δ ct	M	Δ ct	M	M	M
Atp5g1	0.2	–0.21	0.26	–1.20	–2.25	–1.38	0.32	0.66	2.44	0.56	1.89	0.08	–0.42	0.80
Atp6l	2.73	0.79	4.05	0.09	–1.4	–0.02	1.0	–0.59	1.25	1.22	1.48	0.74	2.42	0.98
Cldn7	2.15	–0.40	2.26	0.67	6.26	–0.17	0.29	2.47	4.4	1.96	–0.59	3.51	–2.16	1.94
Grin2d	–4.8	1.65	–2.93	–0.91	5.48	1.90	1.67	–0.97	–6.38	0.76	–3.02	0.27	–0.44	1.17
Kif11	–1.16	–1.26	0.37	–2.59	–5.57	–0.94	–3.64	0.03	–7.49	–0.26	–6.87	0.18	0.52	1.07
PPP1R12A	–3.16	1.51	1.09	2.94	2.62	0.86	0.36	–0.16	–1.43	–0.65	–1.19	–0.03	–0.16	1.26
Pea15	0.56	–0.45	3.97	1.94	0.15	0.25	–0.43	–1.38	0.07	–1.28	–2.23	0.82	–0.27	1.18
Kcna4	–9.51	0.98	–2.1	–1.01	10.05	2.17	1.22	–0.28	–2.33	0.87	–2.92	0.36	0.14	1.02
Prm2	–6.56	–1.55	–0.75	–1.16	1.21	0.92	–0.02	–0.61	–1.96	1.13	–3.28	2.13	0.17	1.33
Tk1	–1.19	–1.73	–3.71	–1.68	1.03	0.32	4.6	–0.03	1.08	–0.23	0.86	0.60	–0.29	0.92
Tubb	–0.89	0.14	0.46	–0.45	0.51	–0.16	0.56	–1.30	–0.05	–0.36	–0.76	–1.35	–0.34	0.57

[*]ΔΔCt is the difference between ΔCt normal control and ΔCt damp-obstructed sample. Fold change in mRNA expression is expressed as $2^{\Delta\Delta Ct}$. ΔCt is the difference in threshold cycles for the sample mRNAs and 18L rRNA. Values for fold-induction varied less than 5% among replicates. The results were derived from the difference of threshold cycle number between normal and damp-obstructed rats after normalization. Standard deviation in the range of 0.3 to 0.5 was obtained but is not shown in this table.

[†]M represents expression ratios of damp-obstructed rats versus normal rats obtained from microarray experiments. The values are logarithmic processed ratios based on 2.

[‡]SD represents overall standard deviations of microarray data

The stomach appeared to be the most severely damaged organ by dampness. Oxidative stress and imbalance of ionic environment directly attacked stomach and caused up-regulation of oxidoreductase activity, electron transport, protease inhibitor activity, and rearrangement of plasma membrane. The ATP metabolic activity was greatly reduced accompanied by the down-regulation of translational apparatus, energy pathways, biosynthesis, membrane structural proteins, and nuclear envelope. It seems the organ is energetically and structurally degenerated in response to dampness.

The lung was also greatly damaged, only slightly less than stomach, probably due to the direct exposure of humidity and heat. The membrane function, nuclear pore, and cytoskeleton were altered. Oxidative stress was high. Many channel and receptor activities were enhanced. Homeostasis was elevated. There seemed to be down-regulation of antigen presentation and processing. The changing of biological processes might indicate a chronic weakening of oxygen-exchanging activity.

The spleen was less affected by oxidative stress despite its displaying up-regulation of plasma membrane. The enhancement of aromatic amino acid metabolism and related melanin activity might be a characteristic indicator of the spleen in response to oxidative stress.

Cellular components of kidney were insignificantly affected by dampness. However, the associated oxidative stress and the requirements for ion transport attacked its internal cellular activity. Most significantly changed was the down-regulation of pathways associated with ATP synthesis and metabolism, which might be the hallmark for the damp-obstructed kidney.

Structural damage to the heart was minor. However, significant elevation of genes associated with apoptosis and down-regulation of immune response indicated that dampness might be a dangerous pathogenic factor that weakens the normal function of the heart. Cell growth and apoptosis were enhanced in liver. Immune response and inflammatory response were also depressed. Cancerous-like growth might be a hallmark for the damp-obstructed liver. The brain also received oxidative stress. Down-regulation of chemokine might be representative for the brain.

RT^2PCR validated the differential expression of genes obtained from microarray experiments and further pinpointed several interesting phenomena of damp-obstructed rats.

ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1 (Atp5g1) is in charge of the transport of protons across a membrane to generate an electrochemical gradient (proton-motive force) that powers ATP synthesis. ATPase, H+ transporting, lysosomal (vacuolar proton pump) 9 kD, on the other hand, depletes ATP storage (Wang and Oster, 1998). The expression levels of these two genes indicated ATP destruction in kidney, spleen, and stomach of damp-obstructed rats. The depletion of ATP in these three organs indicated shortage of energy supply and implies obstruction of qi.

Claudins, including Cldn7, are involved in the formation of tight junctions between epithelial cells. Tight junctions restrict lateral diffusion of lipids and membrane proteins, and thereby physically define the border between the apical and basolateral compartments of epithelial cells (Kominsky *et al.*, 2003). Up-regulation of Cldn7 indicated damage to cell membrane and urgent need for repair.

Glutamate receptors, including Grin2d, are a class of ionotropic glutamate receptors. NMDA channel has been shown to be involved in long-term potentiation, an activity-dependent increase in the efficiency of synaptic transmission thought to underlie certain kinds of memory and learning (New *et al.*, 1998). Up-regulation in the stomach and heart and down-regulation in the other organs indicated disturbance of signal transduction pathways.

Kif11 encodes a motor protein that belongs to the kinesin-like protein family. Members of this protein family are known to be involved in various kinds of spindle dynamics. The function of this gene product includes chromosome positioning, centrosome separation and establishment of a bipolar spindle during cell mitosis (Cochran *et al.*, 2004). It was reported that the ATPase pathway is more similar to conventional kinesin than the spindle motors.

Myosin phosphatase regulates the interaction of actin and myosin downstream of the guanosine triphosphatase Rho. The small guanosine triphosphatase Rho is implicated in myosin light chain (MLC) phosphorylation, which results in contraction of smooth muscle and interaction of actin and myosin in nonmuscle cells (Kiss *et al.*, 2002). Differential expression of this gene directly affects the integrity of the cytoskeleton.

Pea15 is a death effector domain (DED)-containing protein predominantly expressed in the central nervous system, particularly in astrocytes. Reduction of Pea15 expression levels induced apoptosis (Trencia *et al*., 2004).

Kcna4 is a potassium voltage-gated channel protein. Kcna4 may be important in the regulation of the fast repolarizing phase of action potentials in the heart and thus may influence the duration of cardiac action potential (Zhang *et al.*, 2004).

Protamine 2 (Prm2) is involved in fertility (Tanaka *et al.*, 2003). Thymidine kinase is involved in the malignant behavior of epithelial ovarian cancer (Gilles *et al.*, 2003; Berrieman *et al.*, 2004). Further investigation might reveal additional roles of damp obstruction.

As described in the literature, dampness retards middle-energizer and affects the stomach, spleen, and kidney (Huang *et al.*, 2000). Damp obstruction also presents minor symptoms such as the stomach duct and abdominal fullness and distension, a feeling of oppression and discomfort, dizziness, general anxiety, insomnia, weak or fatigued cumbersome limbs, poor appetite, nausea or vomiting, a bitter taste in the mouth, and thick and slimy tongue, frequently with edema (Li, 1976; Chen, 1999). Most symptoms might be explained by the dampness- induced oxidative stress which causes dysfunction of the stomach, insufficient generation of ATP in the kidney, weakening of the heart and lung, and hormonal disregulation in the spleen and brain.

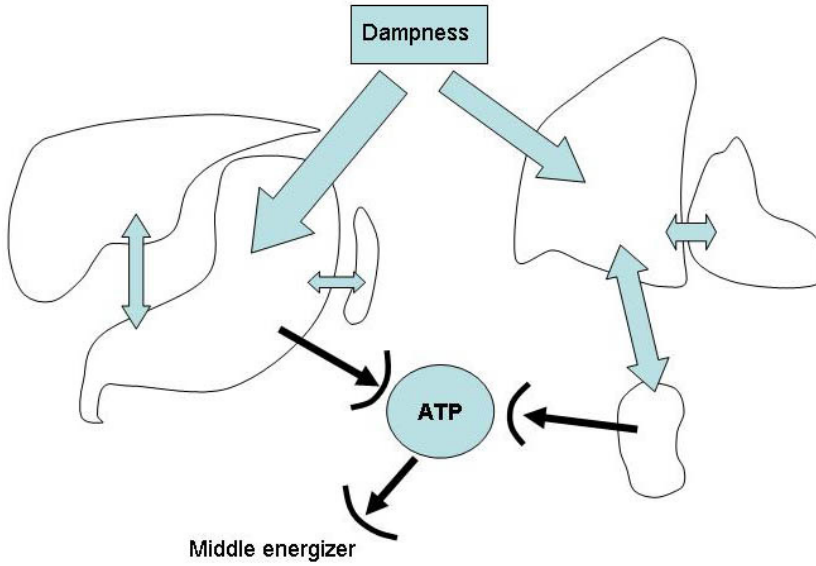

Figure 2. Schematic drawing for the hypothesized pathogenic effects of dampness against major organs leading to the blocking of middle energizer. Organs on the left side are liver (top), stomach and spleen (bottom), representing digestive system. Organs on the right are lung (top), heart (right), and kidney (bottom), representing respiratory and circulatory systems. The degree of obstructing effect of dampness is represented by the thickness of boxed arrow. The functional consequences are represented by solid arrow. Curved lines indicate blocking effect. In the current model, dampness severely attacks the stomach and lung. Dysfunction of the stomach affects liver and spleen and decreases ATP production. Dysfunction of respiratory system eventually affects the kidney function and results in decrease of ATP generation. The overall loss of ATP might serve as a major factor in the obstruction of middle energizer.

Philosophically, the five elements, metal (lung, jin), wood (liver, mu), water (kidney, shui), fire (heart, huo), and earth (spleen and stomach, tu) coordinate by subjugation or restriction to maintain a healthy, physiological state. Normal organ function promotes circulation of vital energy (qi). Introduction of dampness retards the flow of vital energy at middle-energizer, thus inducing dysfunction of spleen and stomach. A reasonable scheme might be proposed in consideration of functional and molecular themes obtained in this study (Fig. 2). At the digestive system, dampness first attacks the stomach, increases oxidative stress, disintegrates structure components, and leads to the dysfunction of the stomach. The dysfunction of the stomach affects the spleen and liver functions. In the circulatory system, lung is directly affected by dampness resulting in enhancing channel and receptor activity and depression of immune response. The function-altered lung weakens the heart and kidney. Brain might be the last organ affected by dampness. We speculate that the imbalance of organ function and down-regulation of ATP biosynthesis might be the major cause for the retardation of qi at middle energizer.

What is the essence of the five elements? Functionally, it is reasonable to hypothesize that tu is correlated to ATP biosynthesis and protein synthesis; shui is correlated to transporter activity and ATP synthesis; huo is associated with immune response and apoptosis; mu is associated with immune and inflammatory responses; and jin is associated with homeostasis and transporter activity.

Dampness might be of little life-threatening significance compared to carcinogenic materials or to direct sunlight. However, it presents a potential oncogenic factor as evidenced by the induction of oncogenic genes in the stomach, kidney, and heart. Early treatment with herbal medicine might be recommended since no known drug is designed for this purpose (Lu and Ma, 1994).

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