Journal of Medicinal Plants Research Vol. 6(11), pp. 2230-2239, 23 March, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.019
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Astragalus polysaccharides induced gene expression profiling of intraepithelial lymphocytes in immune-suppressed mice

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Accepted 22 February, 2012

Astragalus polysaccharides (APS) possess a variety of immunomodulatory activities, but the regulation on mucosal immunity is not fully understood. In this study, immune suppression in mice was induced with cyclophosphamide treatment and APS was used as an intervention and was administrated at dosages of 3 g/kg (APS HD) and 1.5 g/kg (APS LD). Intraepithelial lymphocytes (IELs) were isolated from the intestines. The mRNA expressions from IELs in different groups of mice were detected using gene expression microarray to explore the gene expression profile of IELs, and what are the unique pathways related to low and high dosage of APS.

Key words: Astragalus polysaccharides, mucosal immunity, intraepithelial lymphocytes, gene expression.

INTRODUCTION

The dried root of *Astragalus mongholicus* (Huangqi) has a long history of medicinal use in traditional Chinese medicine. Animal experiments and modern clinical trials have shown that *A. mongholicus* has excellent immunomodulating effects (Li, 1991). Even with the widespread use, a complete understanding of the biological effects and mechanisms regarding *A. mongholicus* has remained largely unknown. The active pharmacological constituents of *A. mongholicus* include various polysaccharides, saponins, and flavonoids. Among these, Astragalus polysaccharides (APS) have been most widely studied. APS might induce the differentiation of splenic DCs with enhancement of T lymphocyte immune function *in vitro* (Liu et al., 2011). Studies have also shown that APS enhances the immunological function of chicken erythrocytes (Jiang et al., 2010a). Moreover, APS can modulate the innate immune response of the urinary tract through inducing increased TLR4 expression *in vitro* (Yin et al., 2010). Apart from these actions, our previous research suggested that regulation of the enteric mucosal immune response could be one of the important pathways for immune modulation by polysaccharides (Zhao et al., 2010). Likewise, we have demonstrated that herbal medicines have a modulating effect on the enteric mucosal immune system (Luo et al., 2010; Xiao et al., 2009).

The mucosal surface of the intestinal tract is the largest body surface in contact with the external environment. It is a complex ecosystem generated by the alliance of gastrointestinal epithelium, immune cells and resident microbiota (McCracken and Lorenz, 2001). Intraepithelial lymphocytes (IELs), as the effector cells of the enteric mucosal immune system, play a multifaceted role in maintaining mucosal homeostasis (Ismail et al., 2009) and may be involved in protective cell-mediated immunity (Mowat et al., 1986). In general, administration of Chinese medicine to individuals is typically done orally, leading to intestinal uptake. Thus, there are extensive interactions between Chinese medicine and intestinal

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tract cells. As IELs are located at this critical interface, they must balance protective immunity with an ability to safeguard the integrity of the epithelial barrier (Cheroutre et al., 2011). So, it is of great interest to detect the genes expression of IELs by APS.

The use of microarrays to evaluate the transcriptome has transformed our view of biology. As with genomic analysis, microarrays are still the best choice for a standardized genome-wide assay that is amenable to high-throughput applications (Git et al., 2010). Effective use of gene expression profiling for biological research has been done to better understand the nature of intestinal development in response to a treatment (Fleet, 2007). Immunosuppressant cyclophosphamide is used to induce the immune-suppressed model (Fang et al., 2006). It is considered an ideal model for investigating many aspects of immunological response. Therefore, by employing immune-suppressed mice, the purpose of the present study was to examine global alterations in gene expression of the IELs in response to APS.

MATERIALS AND METHODS

Experimental animals

Twelve healthy male BALB/C mice, 6-8 weeks old, provided by the China Academy of Chinese Medical Sciences were housed under constant environmental conditions at 22°C and with a 12 h dark-light cycle. The mice were fed a commercially obtained diet and allowed ad libitum access to water. Mice were randomly allocated to four groups: control, cyclophosphamide-induced immune-suppressed group (Model), APS-treated at a high dose (APS HD), and APS-treated at a low dose (APS LD). The approval of the Institutional Animal Ethics Committee was obtained before animal experiments were carried out.

Induction of immune suppression

Immune suppression in the mice was induced with cyclophosphamide (CTX) treatment followed a previously described protocol (Abruzzo et al., 2000; Sun et al., 2002). The mice in the Model, APS HD, and APS LD groups were injected intra-peritoneally with 80 mg/kg cyclophosphamide (Heng Rui Medicine Co., Ltd, China) once each day for three days. The mice in the control group were injected with the same volume of saline.

Administration of APS

APS, a marked drug and proved by the State Food and Drug Administration, China (SFDA No. Z20040086), purchased from Tianjin Cinorch Pharmaceutical Co., Ltd. China, were dissolved with distilled water at a concentration of 250 mg/ml. On the fourth day after induction, all treated mice were given different dosages of orally administered APS once a day in the morning and lasting for 3 days according to their experimental groups: 3 g/kg APS in the APS HD group and 1.5 g/kg APS in the APS LD group. The mice in the control and model groups were administered an equivalent volume of saline.

Isolation of IELs

One day after the last dose of APS, blood was taken through the retro-orbital artery from the mice, and then the mice were sacrificed. The IELs isolation was performed as previously published (Zhao et al., 2010). In brief, the intestines from the duodenum to the ileocecal junction were removed and flushed with Ca^{2+} and Mg^{2+} free HBSS (CMF). Peyer's patches and the mesentery were removed, and the intestines were opened longitudinally and cut into pieces about 10 cm long. The pieces were digested twice for 30 min in CMF containing 10 mM HEPES, 25 mM NaHCO3, 2% FBS, 1 mM EDTA, and 1 mM DTT at 37°C. The eluted cells were collected and passed through a 74 µm nylon mesh to remove undigested tissue pieces. The IELs were subsequently separated from epithelial cells by two centrifugations through a 40/70% Percoll (Pharmacia) gradient at 600 × g for 20 min. The IELs were harvested from the interface between the 40 and 70% Percoll layers.

cRNA labeling

Total RNA was isolated from the IELs using the Trizol extraction method (Invitrogen, Carlsbad, Canada) as described by the manufacturer. mRNAs were amplified linearly using the MessageAmp™ aRNA Kit (Ambion, Inc., Austin, USA) in accordance with the instructions of the manufacturer. cRNA was purified with the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) based on a standard procedure.

Microarray assay

One color format, whole genome mouse Microarray Kit, 4 x 44K (Agilent Technologies) was used in this study. Microarray hybridizations were carried out on labeled cRNAs. Arrays were incubated at 65°C for 17 h in Agilent's microarray hybridization chambers and subsequently washed according to the Agilent protocol. Arrays were scanned at a 5-µm resolution using GenePix Personal 4100A (Molecular Devices Corporation, Sunnyvale, CA).

Statistics and function analysis

All data were analyzed using the SAS9.1.3 statistical package (order no. 195557). Differential gene expression was assessed by ANOVA with the p value adjusted using step-up multiple test correction to control the false discovery rate (FDR). Adjusted p values <0.05 were considered to be significant (Tea et al., 2009). A bioinformatics approach was used to determine the biological context of the large amounts of gene expression data generated by the microarray screen. Gene lists from comparisons showing significant differences were analyzed using the Gene Ontology (GO) classification system, using the DAVID software (http://david.abcc.ncifcrf.gov/tools.jsp) (Dennis et al., 2003; Huang da et al., 2009). Relationships between differentially expressed genes were investigated in pathway analysis using Ingenuity Pathway Analysis (IPA) software (Ingenuity, Redwood City, CA).

RESULTS

Changes of body weight in the mice

From the second day after CTX injection, the mice in the model, APS HD, and APS LD groups showed pathological weight loss. The treated mice all displayed hallmark symptoms of CTX exposure including lassitude and hypoactivity while control animals displayed no

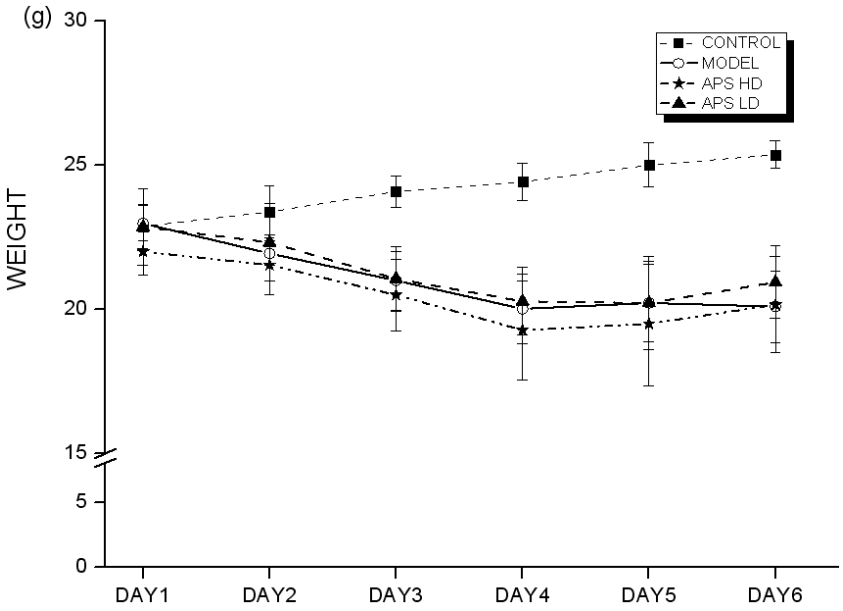

Figure 1. Body weight changes in the mice. From the second day after CTX injection, the mice in model group, the mice in the APS HD and APS LD groups showed pathological weight loss. However, it was noted there was a tendency of weight increase in the APS groups after day 4. The mice in the APS HD and APS LD groups grew faster after administration of APS compared with the model group, although no significant difference was observed in body weight.

Table 1. The significantly expressed genes in each comparison.

Experimental group	Number of gene with significant p values
Control versus model	204
APS HD versus model	22
APS LD versus model	17

obvious symptoms. However, it was noted that there was a tendency of weight gain in APS-treated groups beginning on the day 4. Administration of APS could slow down the tendency of body weights decrease. The mice in the APS HD group and APS LD group grew faster after the administration of APS compared with the model group, although no significant differences in body weight were observed (Figure 1).

Differences in gene expression between groups

The number of genes that were significantly over-expressed or under-expressed is shown in Table 1. Comparisons of gene expression between the control group and model group showed 204 genes to be significantly regulated by CTX. A comparison of APS HD group and model group showed that 22 genes were differentially expressed to a significant. Similarly, 17 genes were observed in APS LD group compared with model group (Table 1). 204 differentially expressed genes between model group and control group clearly separated the model group from the control group. Among these genes, 95 genes were expressed at a significantly higher level in the model group. Using DAVID, 95 up-regulated genes in the model group were found to be involved in functions such as cellular process, binding, and cell projection, whereas 109 down-regulated genes in the model group participated in cellular metabolic processes, catalytic activity, and cell part. The GO terms with up-regulated among 95 genes are indicated in Table 2. The largest group with respect to number of genes (n=55) was the binding in molecular function. The other group with a large number of genes (n=47) was the cellular process in biological process.

Among the 109 down-regulated genes, the GO terms are indicated in Table 3. There were a number of specific GO terms that revolved around the cellular constituent theme of cell and cell part (88 genes). A second prominent group was the cellular process in biological

Table 2. Gene ontology on up-regulated genes in the model group.

GO classification	Specific GO term	Number of genes	p Value
Biological process	Response to stimulus	19	8.1E-3
	Response to stress	12	8.7E-3
	Cellular process	47	2.3E-2
	Cellular developmental process	13	3.4E-2
	Negative regulation of biological process	11	4.5E-2
Molecular function	Protein binding	35	3.6E-3
	Binding	55	5.2E-3
	Ribonucleotide binding	14	2.2E-2
	Purine ribonucleotide binding	14	2.2E-2
	ATP binding	12	2.5E-2
	Adenyl ribonucleotide binding	12	2.7E-2
	Purine nucleotide binding	14	2.9E-2
	Adenyl nucleotide binding	12	3.7E-2
	Purine nucleoside binding	12	3.9E-2
	Nucleoside binding	12	4.1E-2
Cellular constituent	Cell projection	7	2.6E-2

P values reflect statistical significance of each GO term being over-represented.

Table 3. Gene ontology on down-regulated gene in the model group.

GO classification	Specific GO term	Number of genes	p Value
Biological process	Cellular process	69	2.97E-07
	Cellular macromolecule metabolic process	42	3.93E-05
	Cellular component organization	24	7.06E-05
	Macromolecule metabolic process	44	1.24E-04
	Cellular metabolic process	48	1.38E-04
Molecular function	Nucleoside-triphosphatase activity	12	1.57E-04
	Hydrolase activity	24	1.96E-04
	Pyrophosphatase activity	12	2.25E-04
	Hydrolase activity, acting on acid anhydrides, in Phosphorus-containing anhydrides	12	2.38E-04
Cellular constituent	Intracellular part	83	2.75E-12
	Intracellular	83	6.96E-11
	Intracellular organelle	73	3.29E-09
	Organelle	73	3.38E-09
	Nucleus	48	1.73E-07

P values reflect statistical significance of each GO term being over-represented.

process containing 69 genes, which included more specific GO terms such as metabolic process, cellular metabolic process and macromolecule metabolic process. The third group was related to various molecular functions dealing with binding (64 genes).

Also, we identified 9 commonly modulated genes in either APS HD group or APS LD group (Table 4). The genes were regulated in the same direction (towards the normal scale). Those genes must be important genes for elucidating the network how APS affects IELs. To further

Table 4. Shared altered genes between control and model, APS HD and model, APS LD and model.

Gene symbol	Accession #	Control vs. Model (Ratio)	APS HD vs. Model (Ratio)	APS LD vs. Model (Ratio)	Related function or disease
Rnf139	NM_175226	↑(1.57)	↑(1.57)	↑(1.48)	Kidney cancer
2310079N02Rik	AK086714	↑(1.73)	↑(1.54)	↑(1.54)	
Anapc1	AK090134	↑(1.52)	↑(1.47)	↑(1.43)	Gastric cancer
C030015A19Rik	AK028820	↑(1.44)	↑(1.43)	↑(1.45)	
Fbxo3	NM_212433	↑(1.46)	↑(1.40)	↑(1.55)	Oral squamous cell carcinoma
Cpd	AK134736	↑(1.25)	↑(1.33)	↑(1.24)	Stimulate NO production
Derl2	NM_033562	↑(1.26)	↑(1.17)	↑(1.17)	Hepatocellular carcinoma
Ddx17	NM_199079	↓(0.67)	↓(0.73)	↓(0.72)	Dysregulated in cancers
Arl6ip2	NM_019717	↓(0.51)	↓(0.53)	↓(0.54)	

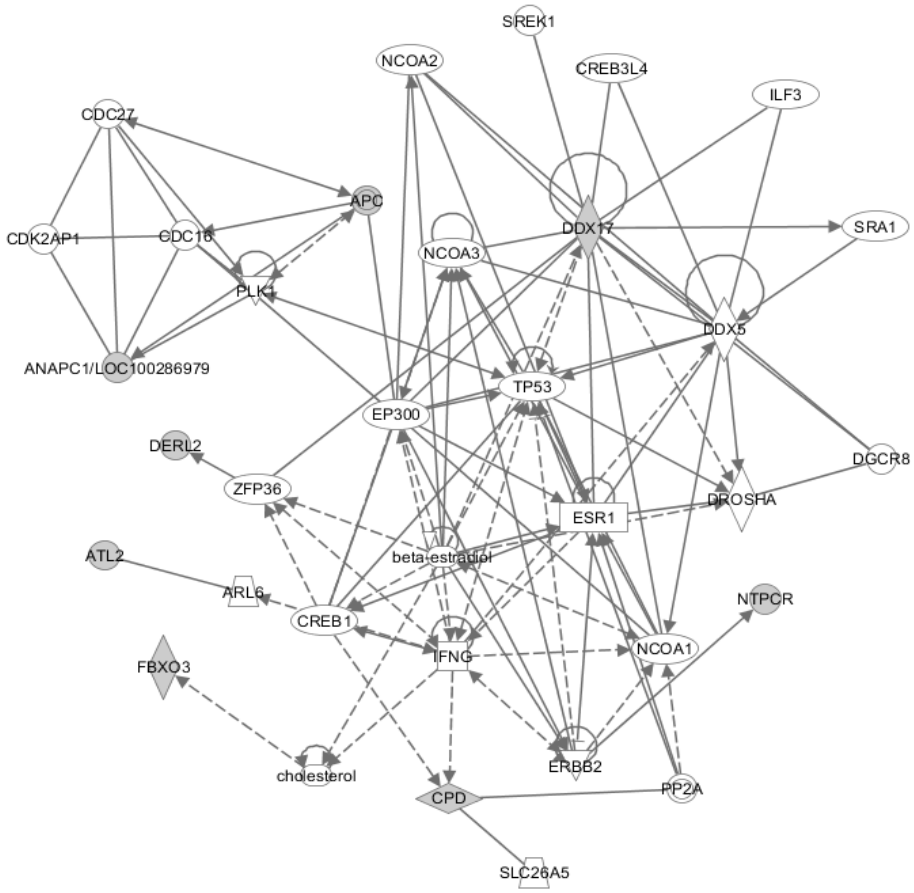

Figure 2. Significant molecular networks of 9 common modulated genes generated by the Ingenuity software. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the gray symbols represent gene entries that occur in our data, while the transparent entries are molecules from the Ingenuity knowledge database, inserted to connect all relevant molecules in a single network. Solid lines between molecules indicate direct physical relationship between molecules; dotted lines indicate indirect functional relationships.

understand the correlations among the candidate genes, bioinformatics analyses were performed using the IPA software, and these analyses led to the identification of biological association networks. As shown in Figure 2,

Table 5. Genes uniquely regulated by APS HD or APS LD.

Gene symbol	Accession #	Control versus model (Ratio)	APS HD versus model(Ratio)	APS LD versus model(Ratio)
Cdc27	NM_145436	↑(1.65)	↑(1.49)	
1110007A13Rik	NM_145955	↑(1.18)	↑(1.17)	
Hmgcr	NM_008255	↑(1.51)	↑(1.42)	
Man1a2	NM_010763	↑(1.44)	↑(1.43)	
D530033C11Rik	NM_030132	↑(1.42)	↑(1.57)	
Ufm1	NM_026435	↑(1.30)	↑(1.30)	
Zcd2	NM_025902	↑(1.25)	↑(1.23)	
Txndc9	NM_172054	↑(1.18)	↑(1.21)	
Zc3h10	NM_134003	↓(0.79)	↓(0.76)	
Sfi1	NM_030207	↓(0.65)	↓(0.72)	
Sidt2	AK081177	↓(0.59)	↓(0.69)	
Nr3c2	NM_001083906	↓(0.4)	↓(0.49)	
Alpi	NM_001081082	↓(0.31)	↓(0.47)	
Tubd1	NM_019756	↑(1.56)		↑(1.43)
AK050250	AK050250	↑(1.20)		↑(1.34)
Hps4	NM_138646	↓(0.78)		↓(0.84)
Ulk1	NM_009469	↓(0.76)		↓(0.73)
Usf2	U01663	↓(0.72)		↓(0.67)
Utrn	NM_011682	↓(0.47)		↓(0.52)
Fosl2	NM_008037	↓(0.42)		↓(0.53)
Hbp1	NM_177993	↓(0.42)		↓(0.41)

the main functionalities for the networks are antigen presentation, cardiovascular disease, cellular development, cancer, cell cycle, cell death, RNA damage and repair, protein synthesis, nutritional disease, developmental disorder, genetic disorder, neurological disease, cellular growth and proliferation.

As we here observe dose-dependent difference of APS, we analyzed special gene expression in IELs. We found that treatment with APS HD led to additional changes in gene expression compared to APS LD, as indicated by the identification of 13 genes regulated specifically by APS HD. In contrast, 9 unique genes were observed to be induced solely by APS LD (Table 5). IPA shows the network about APS HD related genes (Figure 3), and the main functionalities are lipid metabolism, molecular transport, small molecule biochemistry, cancer, cell cycle, cell death, RNA damage and repair, protein synthesis, nutritional disease, developmental disorder, genetic disorder, neurological disease, cellular assembly and organization. The network about APS LD related genes was shown in Figure 4, and the main functionalities given by Ingenuity for the networks are cell morphology, cellular function and maintenance, cell-mediated immune response, antigen presentation, cardiovascular disease, cellular development, cancer, cell cycle, cell death, RNA damage and repair, protein synthesis, nutritional disease, developmental disorder, genetic disorder and neurological disease.

DISCUSSION

The major finding in this study is that APS might play critical in transcriptional regulation of cancer, since the gene expressions of Rnf139, 2310079N02Rik, Anapc1, C030015A19Rik, Fbxo3, Cpd, Derl2, Ddx17, and Arl6ip2, were significantly regulated to normal levels in the APS HD and APS LD treated mice, in which 7 were up-regulated and 2 down-regulated.

Cpd is a type 1 transmembrane protein the cycle between the trans-Golgi network and the plasma membrane. It performs a wide variety of functions, ranging from the digestion of food to the selective biosynthesis of hormones and neuropeptides(Kalinina and Fricker, 2003, Kalinina et al., 2002). Cpd expression is enhanced during inflammatory processes and may stimulate NO production by cleaving Arg from peptide substrates (Hadkar and Skidgel, 2001). Another hallmark of response in the APS-treated mice is the up-regulation of Anapc1, RNF139, Fbxo3, and Derl2 gene expression. A mitotic gene (Fong et al., 2007), The Anapc1 gene was found to contain a long open reading frame of 1944 amino acids, encoding a polypeptide with a calculated molecular mass of 216,087 Da (Starborg et al., 1994). It

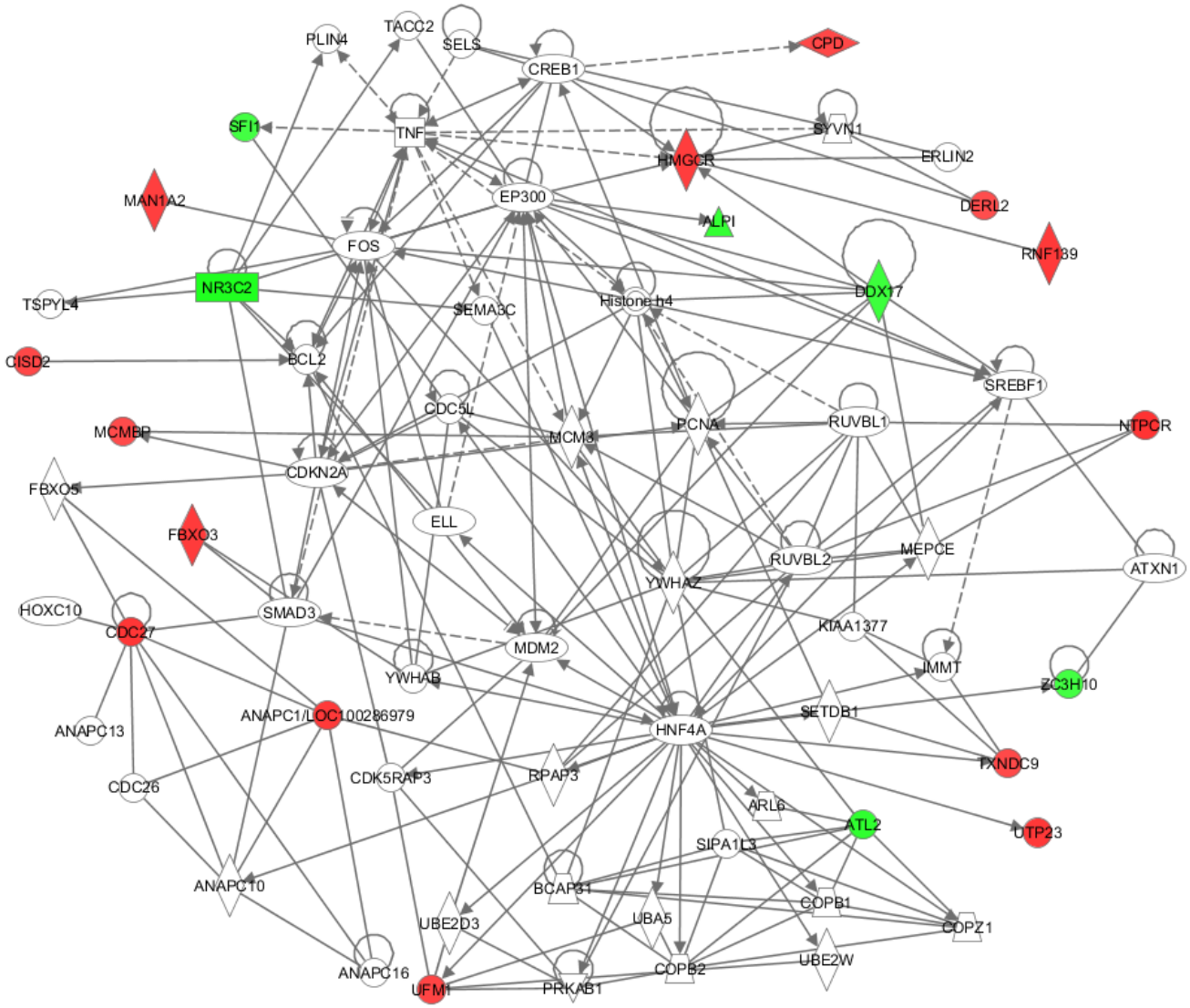

Figure 3. Significant molecular network of APS HD related genes. Genes are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent gene entries that occur in our data, while the transparent entries are molecules from the Ingenuity knowledge database. Red symbols represent up-regulate genes, green symbols represent down-regulate genes. Solid lines between molecules indicate direct physical relationship between molecules; dotted lines indicate indirect functional relationships.

is a centromere-associated protein that appears to have a transient function during mitosis. Anapc1 was identified and shown to be related to an Aspergillus nidulans mitotic checkpoint regulator (Jorgensen et al., 1998). Moreover, Anapc1 may be a possible candidate for causing the chromosomal instability seen in gastric cancer (Lima et al., 2008). RNF139 encodes an endoplasmic reticulum-resident E3 ubiquitin ligase that inhibits growth in a RING- and ubiquitylation-dependent manner (Lee et al., 2010). RNF139 is similar to the Patched family of proteins, with a putative sterol-sensing domain and an extracellular loop capable of interaction with the hedgehog protein (Cho et al., 2005). Chromosomal translocation of this gene may be important in the development of kidney cancer (Gemmill et al., 2002). The Fbxo3 gene may be associated with oral squamous cell carcinoma (OSCC) tumorigenesis and/or progression (Cha et al., 2011), and Fbxo3 can synergistically increase p53 transcriptional activity (Shima et al., 2008). Derl2, a member of the Derlin family, is a putative proto-oncogene and has a direct role in oncogenic transformation (Hu et al., 2007). Increased expression of Derl2 is confirmed in hepatocellular carcinomas (Ying et al., 2001). Taken together, these reports support that the actions of APS might play critical roles in transcriptional regulation in cancer.

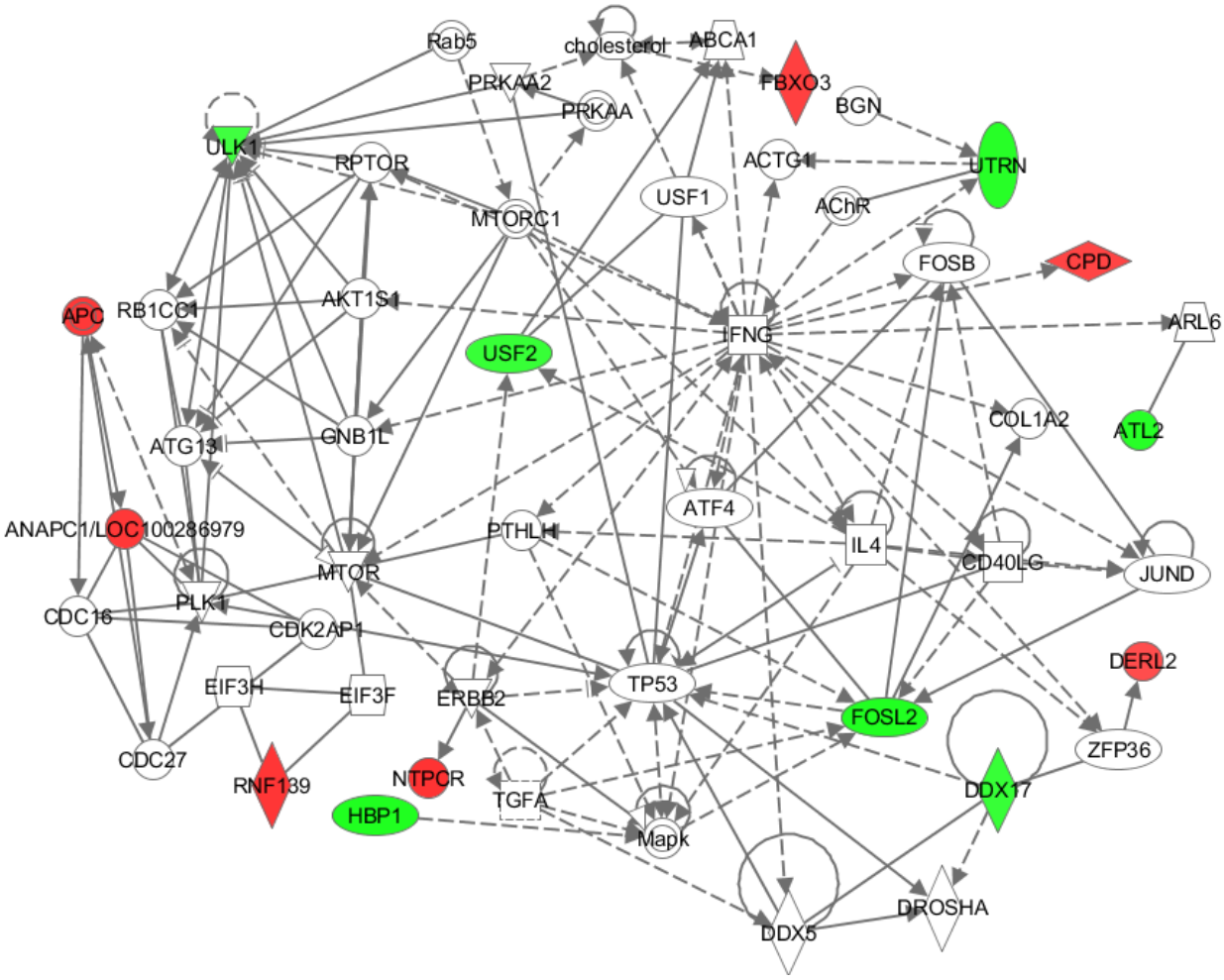

Figure 4. Significant molecular network of APS LD related genes. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent gene entries that occur in our data, while the transparent entries are molecules from the Ingenuity knowledge database. Red symbols represent up-regulate genes, green symbols represent down-regulate genes. Solid lines between molecules indicate direct physical relationship between molecules; dotted lines indicate indirect functional relationships.

The DEAD box RNA helicase Ddx17 plays important roles in multiple cellular processes, including transcription, pre-mRNA processing/alternative splicing, and miRNA processing, which are commonly dysregulated in cancers (Fuller-Pace and Moore, 2011). Blocking Ddx17 acetylation caused cell cycle arrest and apoptosis, revealing an essential role for Ddx17 acetylation (Mooney et al., 2010). The ability of Ddx17 suggests that transcriptional regulation in cancer were at work not only in the up-regulated genes but also in the down-regulated genes. In addition, the Arl6ip2 gene found to be down-regulated in APS-treated groups has been reported in U937 cells exposed to various NO fluxes (Turpaev et al., 2010). However, with few studies of the gene, its relationship with the activity of APS remains unclear. Future studies are needed to test the exact efficacy of interventions that specifically address the processes suggested by the present gene expression studies.

Many of the genes uniquely induced by APS HD or APS LD represented functional families of genes. In the APS HD group, the expressions of Cdc27 and Zc3h10 were induced. In the APS LD group, the expressions of Tubd1 and Fosl2 were modulated. The literature implies that phosphorylation of Cdc27 is involved in TGF-β-induced activation of APC (Zhang et al., 2011), and it is suggested that Cdc27 itself may be a tumor suppressor (Pawar et al., 2010). Zc3h10 inhibits anchorage-independent growth in soft agar, suggesting a tumor suppressor function for this gene (Guardiola-Serrano et al., 2008). The candidate oncogene Tubd1 is associated with breast cancer risk (Kelemen et al., 2009). Fosl2 is a member of the Fos family of AP-1 transcription factors that is often up-regulated in mammary carcinomas. Fosl2

over-expression is associated with a more aggressive tumor phenotype and is probably involved in breast cancer progression in vivo (Milde-Langosch et al., 2008). Interestingly, these 4 unique genes again show the ability to regulate cancer development and progression, a function similar to that of the genes previously mentioned. In addition, Hmgcr, a unique gene affected by APS HD which converts HMG-CoA to mevalonate and catalyzes the rate-limiting step in cholesterol biosynthesis (Ohashi et al., 2003), and upstream stimulatory factor Usf2, a unique gene affected by APS LD which regulates the transcription of genes related to immune response, the cell cycle, and cell proliferation (Bussiere et al., 2010) are needed for further exploration on the association between APS and the activities.

The IPA results showed that the main functionalities for the networks of APS are antigen presentation, cellular development, cell cycle, RNA damage and repair, protein synthesis, cellular growth and proliferation. While the unique pathways in the network of APS LD include lipid metabolism, molecular transport, small molecule biochemistry, and the pathways for APS HD are cell morphology, cellular function and maintenance, cell-mediated immune response. Antigen presentation is the key process for immune response, and it is closely related to RA development (Lebre and Tak, 2009; Wenink et al., 2009; Yanaba et al., 2008). Cell cycle, cellular growth and proliferation, and apoptosis are considered the vital components of various processes including cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death related to RA pathogenesis (Elmore, 2007; Jiang et al., 2010b; Ryu et al., 2010). APS might demonstrate its pharmacological activity via acting on these pathways. However, the differences between the low dosage and high dosage of APS are still waiting for further validation, though there are evidence demonstrating the correlation between lipid metabolism, cell mediated immune response and RA development (Hansel and Bruckert, 2010; Toms et al., 2010). The results further support the findings in DAVID analysis. Though the data presented provides a more comprehensive picture on how APS mediates biological effects on IELs, there is a limitation in this study. RT-PCR did not be conducted for verification. However the clusters of genes and pathway networks are the main purpose to demonstrate the complicated biological networks induced by APS in the mice, and singe gene verification is hard to meet the requirement. Future pharmacological studies are needed to test the efficacy of APS interventions which specifically address the processes suggested by our microarray analysis.

Conclusions

2310079N02Rik, C030015A19Rik, Rnf139, Anapc1, Fbxo3, Cpd, Derl2, Ddx17, Arl6ip2 and the related pathways may be modulated by APS on IELs, and APS might play critical in transcriptional regulations of cancer.

ACKNOWLEDGMENTS

This research is supported in part by the projects from Ministry of Sciences and Technology of China (No. 2009ZX09502-019), National Science Foundation of China, No. 30825047, 30902000, 81001676 and 81001623, E-institutes of Shanghai Municipal Education Commission No E03008, and Key Laboratory of Separation Sciences for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences No. KL1004.

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