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Full Length Research Paper

A regulatory network for human adenocarcinoma

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Human adenocarcinoma (AC) is the most frequently diagnosed human lung cancer and its absolute incidence is increasing dramatically. Our study aimed to interpret the mechanisms of human adenocarcinoma through the regulation network based on differentially expressed genes (DEGs). We used the GSE2514 microarray data to identify human adenocarcinoma differentially expressed genes. Based on these genes and collected regulation datasets, 129 relationships between transcription factor and their target genes were established in AC. Finally, we find some new candidates and relationships, such as IL6 and NFKB1 owning a close connection with AC. Based on a relatively small number of patients, the results will need to be repeated and confirmed in future studies.

Key words: Adenocarcinoma, transcription factors, transcriptome.

INTRODUCTION

Lung carcinomas are usually classified as small-cell lung carcinomas (SCLC) or non-small-cell lung carcinomas (NSCLC). NSCLC is histopathologically and clinically distinct from SCLC and is further subcategorized as adenocarcinomas, squamous cell carcinomas and large-cell carcinomas, of which adenocarcinomas are the most common (Travis et al.,1995).

DNA microarray analysis as a global approach is applied to investigate physiological mechanisms in health and disease (Spies et al., 2002). A high-throughput microarray experiment was designed to analyze genetic expression patterns and identify potential genes to target for AC (Li et al., 2006). Genomic expression profiling evolves as a useful tool to identify novel patho-mechanisms in human cancer (Guo, 2003). These differentially expressed genes found from the expression profiles may play pivotal roles in lung tumorigenesis and may potentially serve as biomarkers in both diagnosis and prognosis of human lung cancer (McDoniels-Silvers et al., 2002; Dong et al., 2011). The development of microarray methods for large-scale analysis of gene expression (Chee et al., 1996) makes it possible to search systematically for molecular markers of cancer classification and outcome prediction in a variety of tumor types (Khan et al., 2001).

The purpose of this study was to propose the hypo-thesis that a transcriptome network can be developed so that a set of transcription factors which regulate the differently expression genes play an important role in which the progress of AC can be identified.

MATERIALS AND METHODS

Data source

Affymetrix microarray data

One transcription profiles of human adenocarcinoma, GSE2514 (Stearman et al., 2005) were obtained from a public functional genomics data repository GEO (http://www.ncbi.nlm.nih.gov/geo/) which are based on the Affymetrix GPL8300 platform data

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(Affymetrix Human Genome U95 Version 2 Array) (Wachi et al., 2005). Only 20 AC chips and 19 control chips are useable. Each pair of samples represents a single patient with AC. One is derived from the cancer cells and the other is from the normal cells.

All patients participating in this study were enrolled in a local colorado multiple institutional review board (COMIRB) approved protocol for use of remnant tissue with anonymization and analysis of specimens and clinical data. All but one of the patients had a history of smoking. Patients range in age from 45 to 73 years of age. Tumors from five males and five females were used in the study. All specimens for microarray analysis were obtained at surgery with nine patients undergoing lobectomy and one wedge resection. Specimens were examined immediately after removal from the patient and grossly visible solid tumor tissue was snap-frozen for RNA extraction. The tumors were all invasive ACs, but five specimens exhibited evidence of bronchoalveolar differentiation at the edge of tumor nests. Most tumors were low to intermediate grade and low stage, although, two stage III tumors were included in the analysis.

Pathway data

Kyoto encyclopedia of genes and genomes (KEGG) is a collection of online databases dealing with genomes, enzymatic pathways and biological chemicals (Kanehisa, 2002). The pathway database records networks of molecular interactions in the cells and variants of them specific to particular organisms (http://www.genome.jp/kegg/). Total 130 pathways, involving 2287 genes, were collected from KEGG.

Regulation data

There are approximately 2600 proteins in the human genome that contain DNA-binding domains and most of these are presumed to function as transcription factors (Wachi et al., 2005). The combinatorial use of a subset of the approximately 2000 human transcription factors easily accounts for the unique regulation of each gene in the human genome during development (Brivanlou and Darnell, 2002).

These transcription factors are grouped into 5 super class families, based on the presence of conserved DNA-binding domains. TRANSFAC database contains data on transcription factors, their experimentally-proven binding sites and regulated genes (Wingender, 2008).

Transcriptional regulatory element database (TRED) has been built in response to increasing needs of an integrated repository for both cis- and trans- regulatory elements in mammals (Jiang et al., 2007). TRED did the curation for transcriptional regulation information, including transcription factor binding motifs and experimental evidence. The curation is currently focusing on target genes of 36 cancer-related TF families. 774 pairs of regulatory relationship between 219 transcription factors (TFs) and 265 target genes were collected from TRANSFAC (http://www.gene-regulation.com/pub/databases.html). 5722 pairs of regulatory relationship between 102 transcription factors (TFs) and 2920 target genes were collected from TRED (http://rulai.cshl.edu/ TRED/).

Combined the two regulation datasets, total 6328 regulatory relationships between 276 TFs and 3002 target genes were collected (Table 1).

Different methods used

Differentially expressed genes (DEGs) analysis

For the GSE2514 dataset, the limma method (Smyth, 2004) was used to identify DEGs. The original expression datasets from all conditions were processed into expression estimates using the RMA method with the default settings implemented in Bioconductor and then construct the linear model. The DEGs only with the fold change value larger than 1.5 and p-value less than 0.05 were selected.

Co-expression analysis

For demonstrating the potential regulatory relationship, the Pearson correlation coefficient (PCC) was calculated for all pair-wise comparisons of gene-expression values between TFs and the DEGs. The regulatory relationships whose absolute PCC are larger than 0.6 were considered as significant.

Gene ontology analysis

The BiNGO analysis (Maere, 2005) was used to identify over-represented GO categories in biological process.

Regulation network construction

Using the regulation data that have been collected from TRANSFAC database and TRED database, we matched the relationships between differentially expressed TFs and its differentially expressed target genes.

Base on the aforementioned two regulation datasets and the pathway relationships of the target genes, we build the regulation networks by cytoscape (Shannon et al., 2003). Base on the significant relationships (PCC > 0.6 or PCC < -0.6) between TFs and its target genes, 129 putative regulatory relationships were predicted between 41 TFs and 92 target genes.

Significance analysis of pathway

We adopted an impact analysis that includes the statistical significance of the set of pathway genes but also considers other crucial factors such as the magnitude of each gene's expression change, the topology of the signaling pathway, their interactions, etc (Draghici et al., 2007). In this model, the impact factor (IF) of a pathway Pi is calculated as the sum of two terms:

$$IF(Pi) = \log(\frac{1}{pi}) + \frac{\sum_{g \in Pi} |PF(g)|}{|E| \cdot N_{de}(Pi)}$$

The first term is a probabilistic term that captures the significance of the given pathway P_i from the perspective of the set of genes contained in it.

It is obtained by using the hyper geometric model in which p_i is the probability of obtaining at least the observed number of differentially expressed gene, N_{de}, just by chance (Tavazoie et al.,1999; Draghici et al.,2003).

The second term is a functional term that depends on the identity of the specific genes that are differentially expressed as well as on the interactions described by the pathway (that is, its topology).

The second term sums up the absolute values of the perturbation factors (PFs) for all genes g on the given pathway P_i.

The PF of a gene g is calculated as follows:

$$PF(g) = E(g) + \sum_{u \in USg} \beta_{ug} \cdot \frac{PF(u)}{N_{ds}(u)}$$

Table1. Regulation data form TRANSFAC and TRED.

Source	Regulation	TFs	Target	Link
TRANSFAC	774	219	265	http://www.gene-regulation.com/pub/databases.html
TRED	5722	102	2920	http://rulai.cshl.edu/TRED/
Total	6328	276	3002	

In this equation, the first term ΔE (g) captures the quantitative information measured in the gene expression experiment. The factor ΔE (g) represents the normalized measured expression change of the gene g. The first term ΔE (g) in the above equation is a sum of all PFs of the genes u directly upstream of the target gene g, normalized by the number of downstream genes of each such gene N_{ds} (u) and weighted by a factor β_{ug}, which reflects the type of interaction: $\beta_{ug} = 1$ for induction, $\beta_{ug} = -1$ for repression (KEGG supply this information about the type of interaction of two genes in the description of the pathway topology). US_g is the set of all such genes upstream of g. We need to normalize with respect to the size of the pathway by dividing the total perturbation by the number of differentially expressed genes on the given pathway, N_{de} (P_i). In order to make the IFs as independent as possible from the technology and also comparable between problems, we also divide the second term in equation 1 by the mean absolute fold change ΔE, calculated across all differentially expressed genes. The result of the significance analysis of pathway was shown in Table 3.

Regulation network between TFs and pathways

To further investigate the regulatory relationships between TFs and pathways, we mapped DEGs to pathways and got a regulation network between TFs and pathways (Figure 2).

RESULTS

Regulation network construction in human adenocarcinoma

We constructed a regulation network of human adenocarcinoma between TFs and its target genes (Figure 1). In this study, PPARG, ETV4, FLI1 and NFKB1 with higher degrees form a local network which suggested that these TFs may play an important role in AC. Besides, the MMP9 was regulated by ERG, FLI1, ETV4 and PPARG; the IL6 was regulated by PPARG, CEBPD and CEBPB which is also observed in our study.

Gene ontology (GO) analysis of the regulation network in human adenocarcinoma

Several GO categories were enriched among these genes in the regulatory network, including positive regulation of cellular process, positive regulation of biological process, response to chemical stimulus and regulation of cell proliferation and so on (Table 2).

Significant pathways in human adenocarcinoma

To identify the relevant pathways changed in AC, we used a statistical approach on pathway level. Significance analysis at single gene level may suffer from the limited number of samples and experimental noise that can severely limit the power of the chosen statistical test. Pathway can provide an alternative way to relax the significance threshold applied to single genes and may lead to a better biological interpretation. So, we adopted a pathway based impact analysis method that contained many factor including the statistical significance of the set of differentially expressed genes in the pathway, the magnitude of each gene's expression change, the topology of the signaling pathway, their interactions and so on. The impact analysis method yields many significant pathways contained leukocyte transendothelial migration, cell adhesion molecules (CAMs), Wnt signaling pathway and so on (Table 3).

Regulation network of TF-pathway

To further investigate the regulatory relationships between TFs and pathways, we mapped DEGs to pathways and got a regulation network between TFs and pathways (Figure 2). In the network, 35 TFs regulated 94 pathways. CEBPB, CEBPD, ETV4, PPARG and so on were shown as hub nodes linked to lots of AC related pathways. EGR1, FLI1and FOS both regulated lots of pathway including Wnt signaling pathway, Focal adhesion, and MAPK signaling pathway and so on.

DISCUSSION

From the result of regulation network construction in AC, we could found that many TFs and pathways closely related with AC have been linked by our method. The genes PPARG, ETV4, FLI1 and NFKB1 are shown as hub nodes in our transcriptome network and some of them were proved related to AC by previous study. The PPARG, ETV4, MMP9, KDR and IL1B were high related to AC proved by previse study. Some evidence also suggests that IL6 and NFKB1 may play important roles to response to AC.

PPARG gene encodes a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes. PPAR-gamma is a regulator of adipocyte differentiation. Published data found both mRNA and protein levels of PPAR-gamma in esophageal adenocarcinoma samples were significantly

Table 2. GO (Gene oncology) biological process analysis.

GO-ID	Object	Count	p-value	corr p-value
48522	positive regulation of cellular process	70	7.57E-27	1.87E-23
48518	positive regulation of biological process	71	4.18E-25	5.15E-22
42221	response to chemical stimulus	58	3.98E-24	3.27E-21
42127	regulation of cell proliferation	43	2.22E-21	1.37E-18
10033	response to organic substance	43	5.99E-21	2.96E-18
51239	regulation of multicellular organismal process	45	3.20E-19	1.31E-16
50794	regulation of cellular process	106	4.00E-19	1.41E-16
48513	organ development	57	5.80E-19	1.79E-16
50793	regulation of developmental process	39	7.42E-19	2.03E-16
31325	positive regulation of cellular metabolic process	42	2.50E-18	5.70E-16

Table 3. Pathway significant analysis.

Database name	Pathway name	Impact factor	Pathway gene in Input (%)	Corrected gamma p-value
KEGG	Leukocyte transendothelial migration	246.32	15.966	2.61E-105
KEGG	Cell adhesion molecules (CAMs)	202.63	14.179	2.03E-86
KEGG	Wnt signaling pathway	39.63	5.263	2.50E-16
KEGG	Adipocytokine signaling pathway	15.68	4.478	2.59E-06
KEGG	TGF-beta signaling pathway	11.47	14.943	1.30E-04
KEGG	Complement and coagulation cascades	11.28	17.391	1.55E-04
KEGG	Focal adhesion	11.01	18.227	1.99E-04
KEGG	ECM-receptor interaction	10.03	23.81	4.87E-04
KEGG	PPAR signaling pathway	8.38	15.714	0.0021442
KEGG	Tight junction	6.05	12.593	0.0166372

higher than normal samples. Increased PPAR-gamma expression may play an important role in the development and progression from normal cells to esophageal adenocarcinoma (Wang et al., 2011). The vast majority of colorectal cancer is an adenocarcinoma. Ligand activation of PPARgamma in colon cancer cells causes a considerable reduction in linear and clonogenic growth, increased expression of carcinoembryonic antigen and the reversal of many gene expression events specifically associated with colon cancer. These results indicate that the growth and differentiation of colon cancer cells can be modulated through PPARgamma (Sarraf et al., 1998).

ETV4 is an ets-oncogene family transcription factor. ETV4 was shown to upregulate multiple matrix metallo-proteinase (MMP) genes and contribute to the malignant phenotype of cancer cells by inducing invasive and metastatic activities (Shindoh et al., 2004). Previous research showed that the ERK-ETV4-MMP-1 axis is upregulated in oesophageal adenocarcinoma cells and is a potentially important driver of the metastatic pro-gression of oesophageal adenocarcinomas (Keld et al., 2010). Further research also found that expression of MMP-1 and matrilysin correlated significantly with E1AF expression in human colorectal cancer. The results of this study suggest that E1AF plays a key role in the progression of colorectal cancer (Horiuchi et al., 2003).

E2F4 gene encodes a protein of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses. Examination of patients with human colon cancer found that expression of E2F4 was upregulated and the cell apoptosis was suppressed. These data suggest that E2F-4 gene overexpression plays a role in the development of colorectal tumors (Mady et al., 2002). The mutations of the serine (AGC) 13 repeats within the E2F-4 gene were found only in the squamous cell carcinoma, whereas such alterations were not detected in any of the adenocarcinomatous. This suggests that E2F-4 might be implicated in the trans-formation of adenocarcinoma into squamous cell carcinoma, though, the underlying mechanism needs to be studied (Woo et al., 2000).

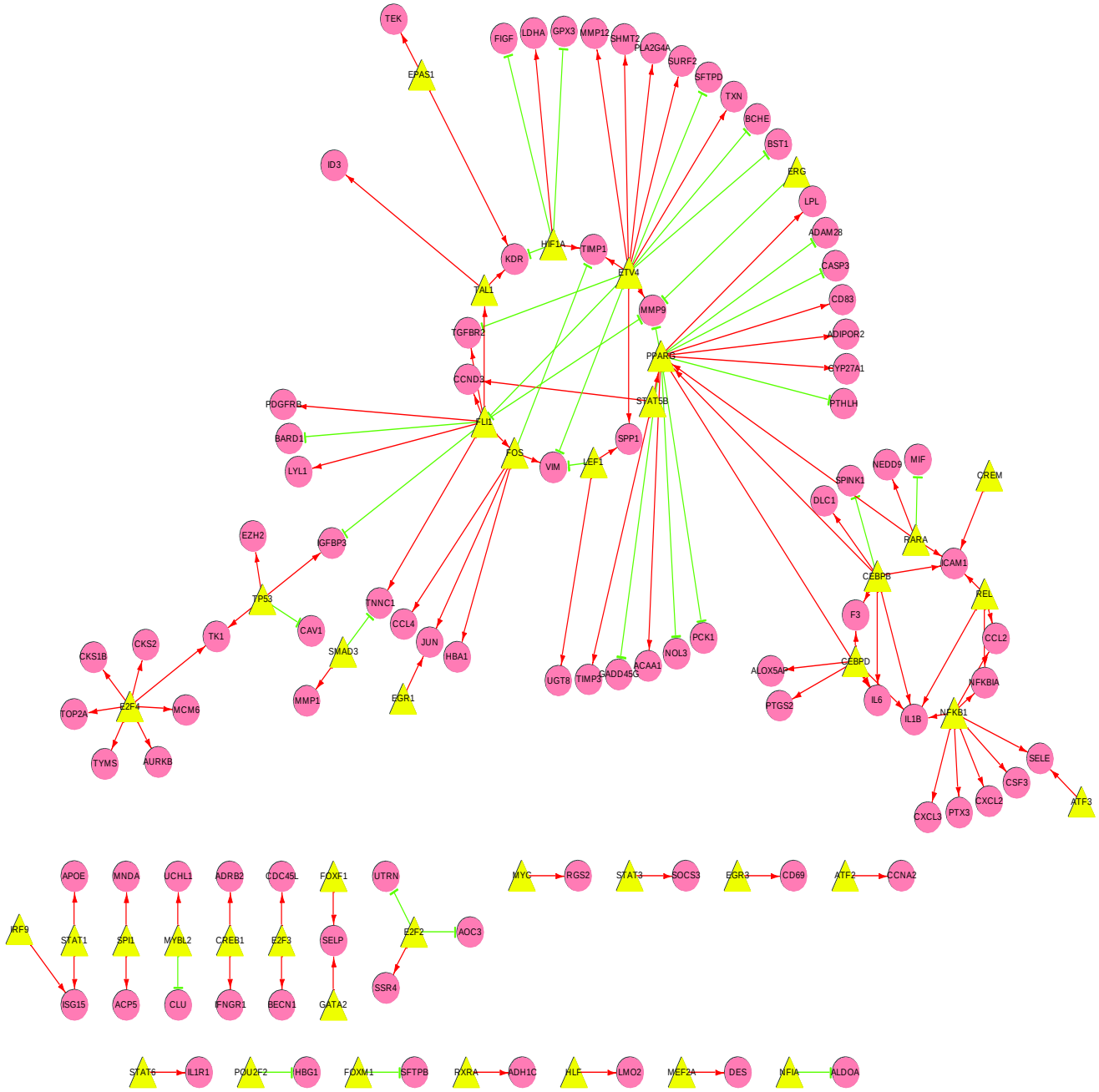

Figure 1. Regulation network construction in human adenocarcinoma.

MYC encoded a multifunctional nuclear phosphor-proteinthat plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Myc-associated protein with JmjC domain (MAPJD) is a novel member of the MYC transcriptional complex and its activation is a common feature of lung cancer, selective suppression of this pathway could be a promising therapeutic target for treatment of lung cancers (Suzuki et al., 2007). Altered apoptotic balance happened in the pathogenesis of human adenocarcinomas and MYC family genes might affect oncogenesis through distinct sets of targets, in particular implicating the importance of transcriptional repression (Kim et al., 2006).

MMP9 belongs to the matrix metalloproteinase (MMP) family which are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and

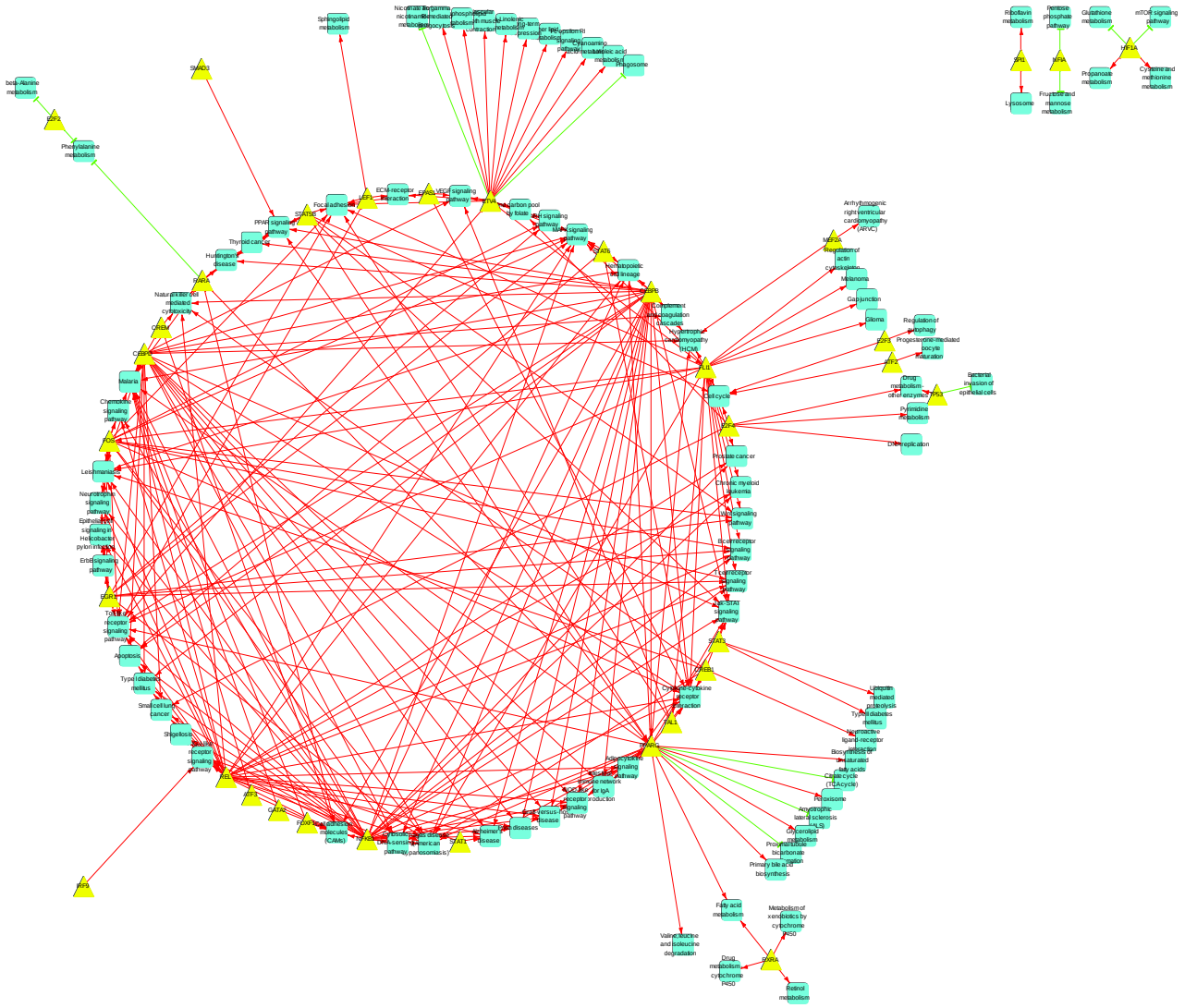

Figure 2. Regulation network of TF-Pathway.

tissue remodeling, as well as in disease processes, such as arthritis and metastasis. The ischemia-reperfusion (I/R) injury incurred during liver surgery can lead to cellular dysfunction and elevations in proinflammatory cytokines and matrix metalloproteinases. Previous study found that hepatic I/R-induced elevations in MMP9 contribute to the growth of metastatic colorectal carcinoma in the liver and that postresection MMP9 inhibition may be clinically beneficial in preventing recurrence following hepatic surgery (Nicoud et al., 2007). MMP family including MMP9 may be relevant with carcinogenesis, development and metastasis of adenoid cystic carcinoma. Furthermore, MMP9 would be required in the initial steps of invadopodia formation (Nascimento et al., 2010).

KDR (VERFR2) encodes one of the two receptors of the VEGF which is a major growth factor for endothelial cells. It functions as the main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis and sprouting. Based on analysis of salivary adenoid cystic carcinoma (SACC) patients, positive correlation between expression of VEGFR2 and nerve invasion and vessel metastasis of SACC was found. This indicates that VEGFR2 play important roles in the invasion and metastasis of SACC (Nong et al., 2010). VEGFR2 showed a higher expression level in metastasis than primary tumors of medullary thyroid carcinoma. This indicated the activation of VEGFR2 may play an important role in metastasis of medullary thyroid carcinoma (Rodriguez-Antona et al., 2010).

IL1B encodes a protein of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular

activities, including cell proliferation, differentiation, and apoptosis. IL-1B is a potent inhibitor of gastric acid secretion. Studies suggest that it is a key determinant in gastric adenocarcinoma. Furthermore, IL-1B promoter variants T31C and C511T, contribute to the risk of developing gastric cancer based on the investigation both Chinese and Japanese population (Yang et al., 2004; Ikehara et al., 2006). Advanced studies also proved an association of 1L1B gene polymorphisms at the two sites with lung cancer risk. Patients who have homozygote genotypes were more likely to have a mutation in the p53 gene which is common in many cancers (Zienolddiny et al., 2004).

IL6 encodes a cytokine that functions in inflammation and the maturation of B cells. IL6 protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through its receptor. Mutant EGFR could activate the gp130/JAK/STAT3 pathway by means of IL-6 upregulation in primary human lung adenocarcinomas. In addition, reduction of IL-6 levels by RNA interference led to a decrease in tumorigenesis. All those make this pathway a potential target for cancer treatment (Gao et al., 2007).

NFKB1 is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. Activated NFKB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions. Analysis of several pancreatic ductal adenocarcinoma cell lines displayed dramatically elevated levels of subsets of the non-canonical NFkappaB target genes CCL19, CCL21, CXCL12, CXCL13 and BAFF. This demonstrates that the non-canonical NFkappaB pathway is constitutively active and functional in pancreatic cancer cells (Wharry et al., 2009).

From the result of regulation network between TFs and pathways in OA, we could found that there are many pathways such as Leukocyte transendothelial migration, Wnt signaling, which are closely related with OA have been linked by our method.

Leukocyte transendothelial migration is the pathway that leukocyte migrate from the blood into tissues. This process is vital for immune surveillance and inflammation. During this diapedesis of leukocytes, the leukocytes bind to endothelial cell adhesion molecules (CAM) and then migrate across the vascular endothelium. In endometrioid adenocarcinoma, activated leukocyte cell adhesion molecule (ALCAM) expression was significantly increased in high-grade tumors and cases with myometrial invasion (Liang et al., 2011). In pancreatic ductal adenocarcinoma (PDAC), the expression of ALCAM is also altered and its serum levels are increased. *In vitro*, ALCAM silencing using RNAi had no effects on growth or invasion of pancreatic cancer cells but reduced cell adhesion and induced chemo-resistance (Hong et al., 2010). All those studies proved leukocyte migration involved in the development of different adenocarcinomas.

Wnt signaling play a key role in the developmental and homeostatic processes including stem cell maintenance, growth and cell fate specification, cell polarity and migration. In the Wnt/β-catenin cascade, it's mainly means the regulation of ubiquitin-mediated degradation of the crucial transcriptional regulator β-catenin (Tauriello and Maurice, 2010). Activation of Wnt/β-catenin signaling resulted in mouse prostatic intraepithelial neoplasia (mPIN). Furthermore, the activation of both SV40-large T-antigen (Tag) and the Wnt/β-catenin pathway resulted in invasive prostate adenocarcinoma. This indicates that Wnt/β-catenin signaling has an important role in the progression of prostate adenocarcinoma (Yu et al., 2010). Wnt-antagonist Dickkopf gene (DKK) 3 involved in embryonic development through its inhibition of the WNT signaling pathway. Knockdown of DKK3 expression by DKK3 SiRNA transfection led to the detachment of lung adenocarcinoma cells from the bottom of the culture plate and caused apoptosis (Jung et al., 2010).

Adipocytokine signaling pathway may play an important role in OA. Resistin levels were significantly higher in colon cancer patients while leptin serum levels were significantly lower as compared to controls. Leptin levels decreased gradually with tumor stage and aggressiveness. Taken together, these results of this study suggest that adipokines, in particular resistin and leptin may be involved in development and progression of colon cancer (Salageanu et al., 2010). Adipo-R1 and Adipo-R2 are two receptors of adiponectin (ApN) which is a 30 kDa adipocytokine. Adipo-R1 and Adipo-R2 immunoexpression was only found in gastric cancer and in intestinal metaplasia areas near the tumours, but not in normal tissues (Barresi et al., 2009). In addition, the two receptors Adipo-R1 and Adipo-R2 of ApN also have been reported in colorectal cancer tissue and cell line, and the expression of ApN was highly associated with the colorectal tumors (Barresi et al., 2009). Therefore, further studies of the roles adipocytokine played in different adenocarcinomas may be beneficial for anticancer therapies.

The basic understanding of the mechanisms underlying AC is important. A deeper understanding of transcription factors and their regulated genes remain an area of intense research activity in futures. Our regulation network is useful in investigating the complex interacting mechanisms of transcription factors and their regulated genes. However, further experiments are still needed to confirm the conclusion.

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