

Comparative transcriptome analysis between metastatic and non-metastatic gastric cancer reveals potential biomarkers

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Abstract. The transcriptome of metastatic gastric cancer (GC) was compared to that of non-metastatic GC to identify metastasis-related biomarkers. The gene expression dataset GSE21328, comprising 2 metastatic GC samples and 2 non-metastatic GC samples, was downloaded from the Gene Expression Omnibus database. Differential expression analysis was performed with the package limma of Bioconductor to identify differentially expressed genes (DEGs). Gene Ontology (GO) enrichment analysis was performed to identify significantly altered biological functions. In addition, the transcriptional regulatory and protein-protein interaction networks were constructed with information from the UCSC genome browser and STRING database, respectively, followed by functional enrichment analysis of all of the genes in these two networks. A total of 584 DEGs were identified, of which 175 were upregulated and 409 downregulated. Clustering analysis confirmed that these genes can distinguish metastatic from non-metastatic GC. Upregulated genes were enriched for the xenobiotic metabolic process, while downregulated genes were enriched for immune response and related pathways. Among the 584 DEGs, six genes (DAND5, EGR2, FOXD1, LMO2, PRRX2 and STAT1) were shown to encode transcription factors, which were used to establish the transcriptional regulatory network with 169 target genes, forming 175 nodes. The proteins of this network were significantly enriched for the process of negative regulation of cell differentiation. In conclusion, this study identified a range of DEGs in meta-

static GC, which may enhance our current knowledge on this disease. Among these genes, *STAT1* and *EGR2* may constitute potential biomarkers of GC metastasis.

Introduction

Gastric cancer (GC) is the fourth most common cancer worldwide. It causes ~800,000 deaths/year, which renders it the second leading cause of cancer-related mortality after lung cancer (1). Metastasis occurs in 80-90% of individuals with GC, with a six-month survival rate of 65% for patients diagnosed at the early stages, and <15% for those diagnosed at late stages. Therefore, metastasis-related biomarkers are necessary to allow early diagnosis and development of targeted therapies.

A number of studies have demonstrated that diverse signaling pathways are involved in the metastasis of GC. Yonemura *et al* (2) demonstrated that cancer cells producing VEGF-C induces proliferation and dilation of lymphatic vessels, resulting in the invasion of cancer cells into the lymphatic vessel and lymph node metastasis. Shimizu *et al* (3) further showed that inhibition of VEGFR-3 signaling inhibits lymph node metastasis of GC cells. Xu *et al* (4) found that expression of receptors for advanced glycation end-products is closely associated with the invasive and metastatic activity of GC cells. The tumor microenvironment also appears to considerably affect metastasis. Upregulation of manganese superoxide dismutase was found in metastatic GC, which may be associated with the reactive oxygen status of the gastric tumor microenvironment (5). The epithelial cell adhesion molecule E-cadherin also plays a role in tumorigenesis and metastasis of GC cells (6). Given the poor outcome of patients with metastatic GC, additional research is necessary to unveil the molecular mechanisms underlying metastasis, and thus provide useful biomarkers for early diagnosis and treatment.

Microarray technology is an appropriate tool to investigate the global gene expression changes in cancers such as GC. Hippo *et al* (7) adopted this technology to study the global gene expression profiles in GC. Kang *et al* (8) identified differentially expressed genes (DEGs) in drug-resistant GC cells. Yamashita *et al* (9) carried out a genome-wide screening for methylation-silenced genes using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarrays.

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By now, massive microarray data have been acquired. We considered that a comprehensive exploration of existing datasets with bioinformatic tools may provide novel insights into GC metastasis. Therefore, in the present study, a comparative analysis between metastatic and non-metastatic GC gene expression data was performed to identify DEGs, which may allow to reveal the molecular mechanisms underlying metastasis and provide potential biomarkers for the early diagnosis of this disease.

Materials and methods

Microarray data and pretreatment. The gene expression dataset GSE21328 was downloaded from the Gene Expression Omnibus database (10), and comprised 2 metastatic stomach cancer samples and 2 non-metastatic stomach cancer samples (11). The platform in which the data were acquired was the Agilent-014850 Whole Human Genome 4x44K G4112F microarray (feature number version).

Raw data were \log_2 transformed. Probes were mapped to genes according to the annotation file. The average expression level for each gene was calculated from the expression data for all probes corresponding to the same gene. Normalization of the data was performed with the Affy package of Bioconductor (12).

Identification of DEGs. The Bioconductor package limma (13) was chosen for differential expression analysis. As cut-offs, we used the \log (fold-change) >1.5 and p -value <0.05 . Hierarchical clustering of the DEGs was performed on median-centered, \log_2 -transformed data using the Cluster software (14), and was subsequently visualized using Treeview software (15).

Functional enrichment analysis. Gene Ontology (GO) enrichment analysis was performed for upregulated and downregulated genes with DAVID (16). Terms with false discovery rate (FDR) <0.05 were retained, and then compared between the two groups.

Transcriptional regulatory network analysis. A transcriptional regulatory network was constructed for DEGs with information from the UCSC genome browser (<http://genome.ucsc.edu>) (17). GO and Kyoto Encyclopedia of Genes and Genomes (KEGG; 18) enrichment analysis were performed for the nodes in the network in order to identify proteins and pathways that were significantly enriched in metastasis. FDR <0.05 was set as the cut-off.

Construction of a protein-protein interaction (PPI) network. A PPI network was constructed for DEGs using information from the STRING database (<http://string-db.org/>). The interactions with score >0.7 were retrieved from the database and then visualized using the Cytoscape software platform (19). The proteins in the network were defined as the nodes and the degree of a node corresponds to the number of interactions of a protein. Functional enrichment analysis was applied on hub nodes with degree of >5 . The Clustering with Overlapping Neighborhood Expansion (ClusterONE) plugin from Cytoscape (20) was used to extract functional modules from

the entire network. Number of node ≥ 10 and p -value <0.05 were set as the cut-offs.

Results

DEGs. The microarray dataset GSE21328 included 12,116 gene expression values. Normalization of these data was satisfactory (Fig. 1). A total of 584 DEGs were then identified for metastatic stomach cancer, of which 175 were upregulated and 409 were downregulated. The clustering analysis result is shown in Fig. 2, indicating these genes may be used to distinguish between metastatic and non-metastatic GC.

Functional enrichment analysis. Upregulated genes were enriched for the GO term 'xenobiotic metabolic process'. Downregulated genes were enriched for 'immune response', 'response to virus', 'antigen processing and presentation', and 'cell morphogenesis involved in differentiation'. In addition, a considerable portion of DEGs was predicted to locate at the extracellular space (Table I).

Transcriptional regulatory network. A total of 215 transcription factors (TFs) and 21,4607 TF-target gene interactions are included in the UCSC database. Out of the 584 DEGs, 6 downregulated genes were found to encode TFs. These TFs were: DAN domain family member 5 (DAND5), which is a BMP antagonist, early growth response 2 (EGR2), forkhead box D1 (FOXD1), LIM domain only 2 (LMO2), paired mesoderm homeobox protein 2 (PRRX2) and signal transducer and activator of transcription 1 (STAT1). No upregulated TFs were detected. A transcriptional regulatory network was constructed on the 6 TFs, and their 169 target genes (Fig. 3), forming 175 nodes (proteins) and 285 edges (interactions). Functional enrichment analysis showed that the proteins in this network are significantly enriched for the GO term 'negative regulation of cell differentiation' (FDR <0.05). This category included proteins such as noggin (NOG), LIM domain only 2 (LMO2), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), delta-like 3 (DLL3), tumor protein p63 (TP63), Toll-like receptor 3 (TLR3), nuclear receptor subfamily 2, group E, member 1 (NR2E1), parathyroid hormone-like hormone (PTH1LH), brain-derived neurotrophic factor (BDNF), PR domain containing 6 (PRDM6), inositol polyphosphate-5-phosphatase (INPP5D), chordin (CHRD), and twist basic helix-loop-helix transcription factor 2 (TWIST2).

PPI network. Fig. 4 shows the PPI network of DEGs in metastatic GC, which contained 191 nodes and 474 edges. The number of downregulated proteins was higher than that of upregulated proteins. Moreover, downregulated proteins were often observed as components of the same module, such as in module 1 in Fig. 5. A total of 6 modules were identified from the entire network (Fig. 5) using ClusterONE. Module 1 comprised 32 downregulated genes products and 245 interactions. Functional enrichment analysis showed that proteins of this module were significantly enriched for immune functions, such as immune response (FDR <0.05) and antigen processing and presentation of peptide antigen via MHC class I (FDR <0.05). No significant enrichment for GO terms was observed in the remaining 5 modules. However, STAT1

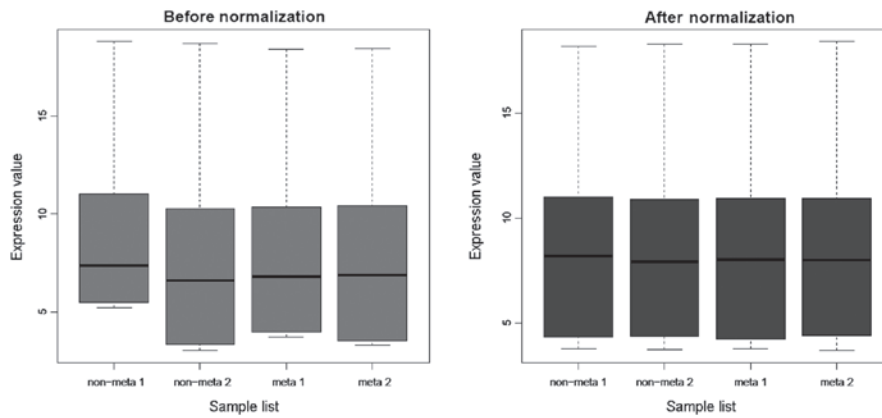


Figure 1. Boxplots of gene expression data before (left) and after normalization (right). The medians (horizontal black lines) are nearly at the same level, indicating a good performance of normalization. Meta, metastasis.

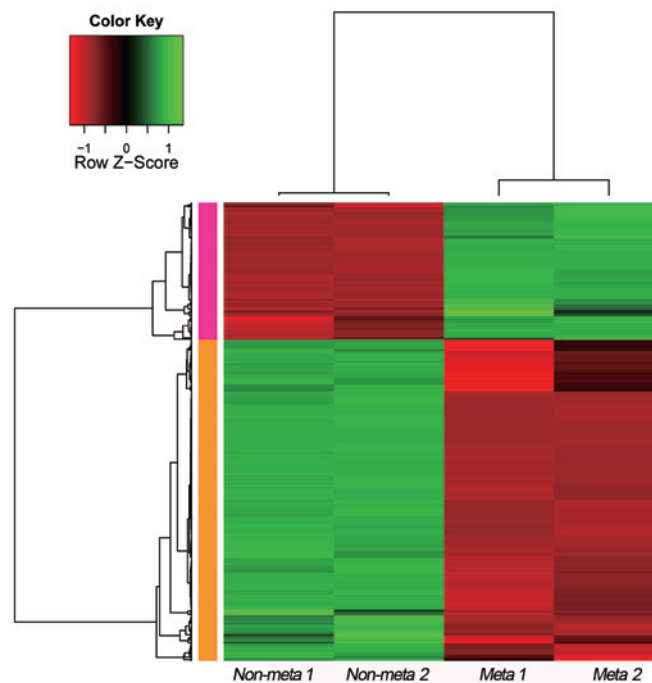


Figure 2. Clustering analysis of differentially expressed genes. Data were derived from the \log_2 transformation for each gene. Red, high expression; green, low expression, according to z scores. Meta, metastasis.

Table I. GO enrichment analysis results for upregulated and downregulated genes.

Category	GO term	Count	P-value	FDR
Upregulated genes	0006805-xenobiotic metabolic process	5	3.88E-02	4.64E-02
Downregulated genes	0006955-immune response	45	8.13E-09	6.86E-09
	0009615-response to virus	16	7.17E-06	6.05E-06
	0019882-antigen processing and presentation	13	1.56E-04	1.31E-04
	0000904-cell morphogenesis involved in differentiation	17	5.50E-02	4.77E-02
	0005615-extracellular space	35	3.91E-04	1.96E-03
	0044421-extracellular region part	42	1.38E-03	6.91E-03
	0005576-extracellular region	69	3.08E-03	1.55E-02
	0042611-MHC protein complex	9	5.05E-03	2.54E-02
	0009986-cell surface	21	8.53E-03	4.29E-02

Count, the number of differentially expressed genes. FDR, false discovery rate; GO, Gene Ontology.

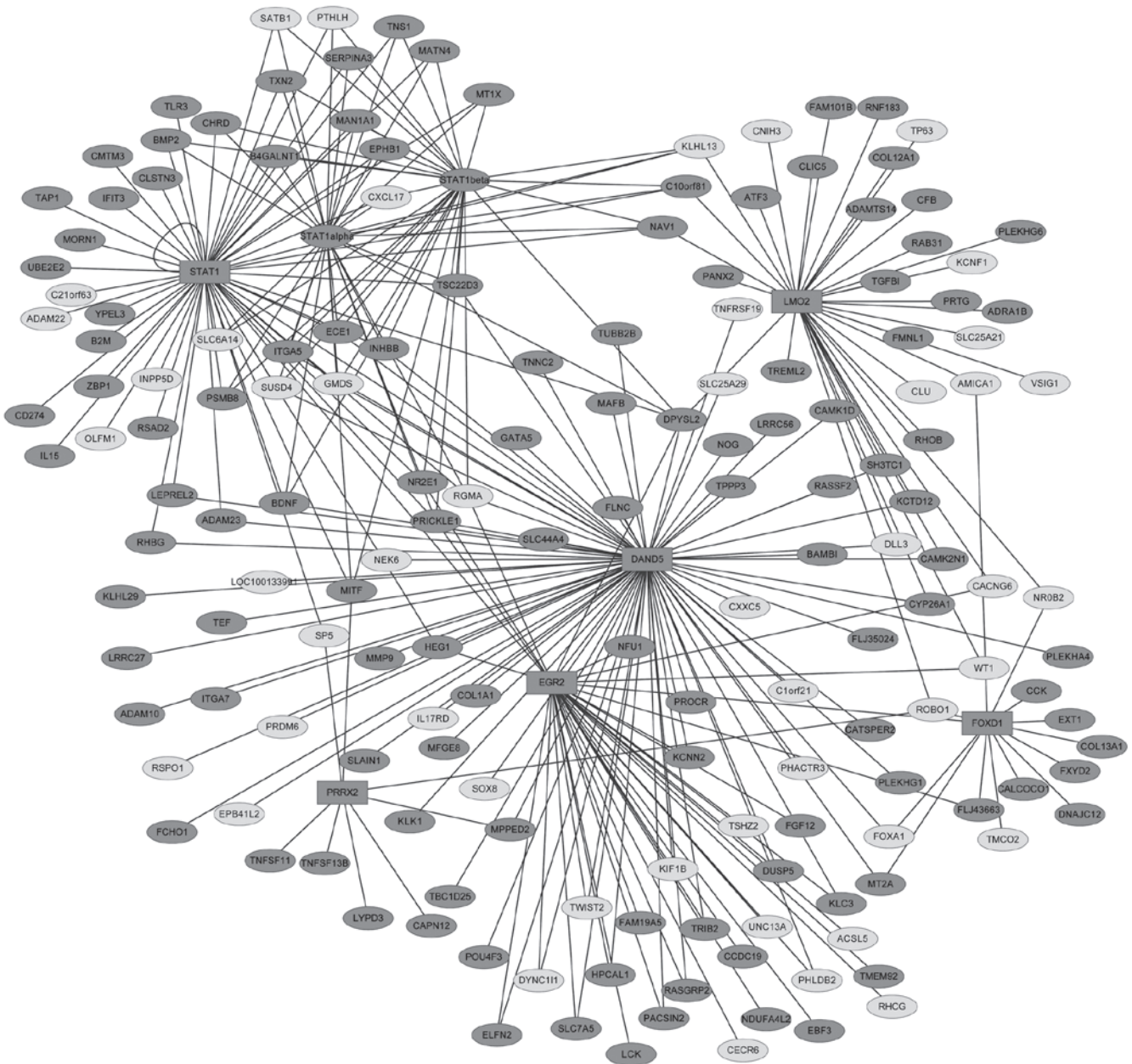


Figure 3. The transcriptional regulatory network of differentially expressed genes in metastatic gastric cancer. Dark rectangles represent transcription factors (n=6), gray ellipses upregulated target genes (n=49), and dark ellipses downregulated target genes (n=120).

was found to be part of two modules (1 and 2), suggesting that this protein may have a role in the development of metastatic GC.

Discussion

In the present study, a total of 584 DEGs were identified in metastatic GC, of which 175 were upregulated and 409 downregulated. GO enrichment analysis revealed that the downregulated genes were significantly enriched for immune response and relevant pathways. According to the transcriptional regulatory network analysis, 6 DEGs were found to be TFs, associated with 169 predicted targets. Functional enrichment analysis revealed that the genes in this network are enriched for negative regulation of cell differentiation. In addition, 6 functional modules were extracted from the PPI

network; of these modules, module 1 showed a significant overrepresentation for immune response and relevant biological functions.

Immune response is closely related to cancer, while immune escape is a critical gateway to malignancy (21). The study by Maehara *et al* (22) confirmed that there is a negative correlation between lymph node metastasis and dendritic cell infiltration in GC. In agreement with previous studies, a number of downregulated genes in metastatic GC were found to be involved in immune response, as well as antigen processing and presentation of peptide antigen via major histocompatibility complex (MHC) class I (Fig. 5, module 1). Downregulation of human leukocyte antigen (HLA) class I antigen processing molecules is considered to relate to renal cell carcinoma (23). Cabrera *et al* (24) found that HLA class I expression in metastatic melanoma correlates to tumor development during

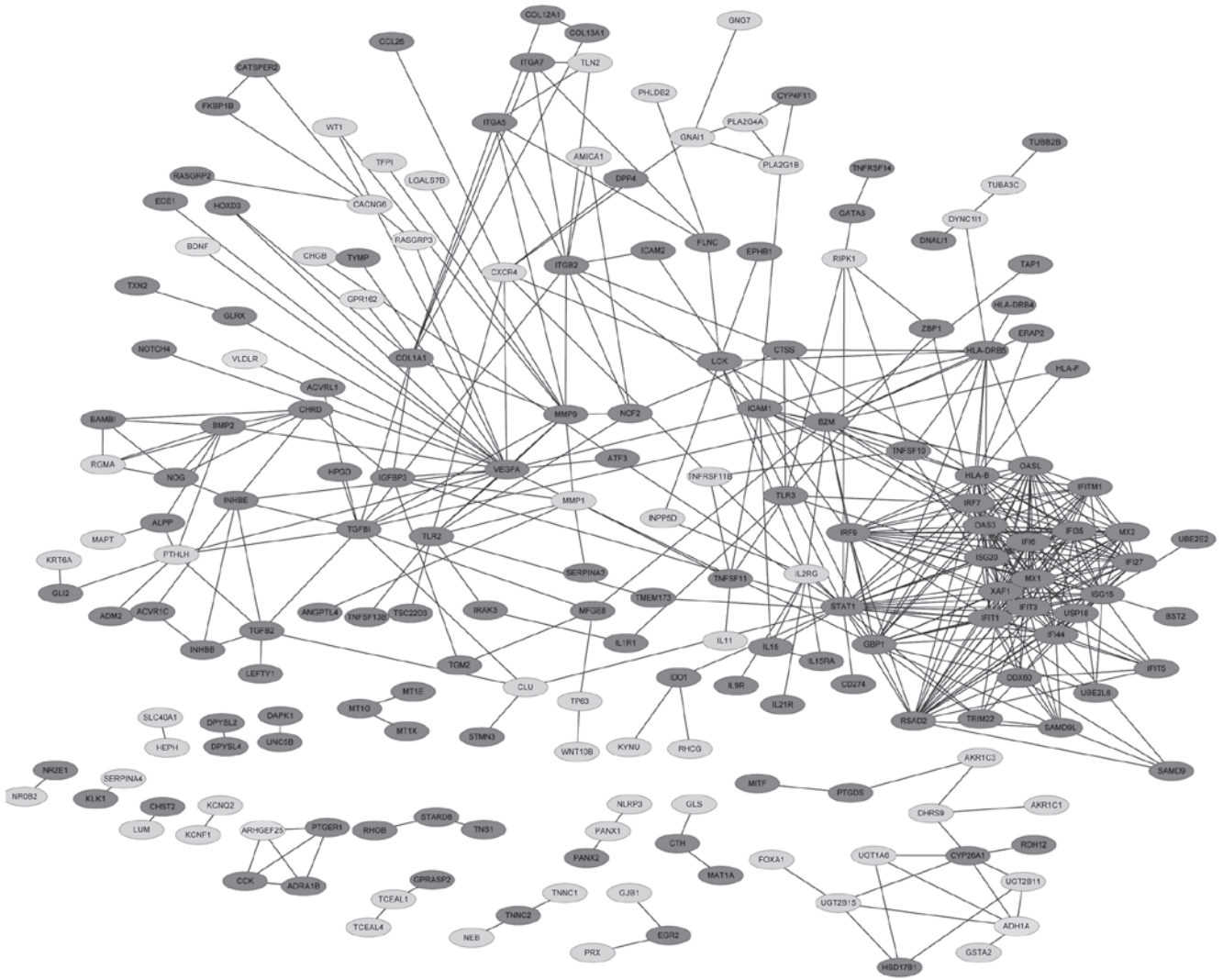


Figure 4. The protein-protein interaction network of differentially expressed gene products in metastatic gastric cancer. Downregulated proteins in metastatic GC are in dark (n=133), upregulated proteins in gray (n=55), and non-differentially expressed ones in light gray (n=6).

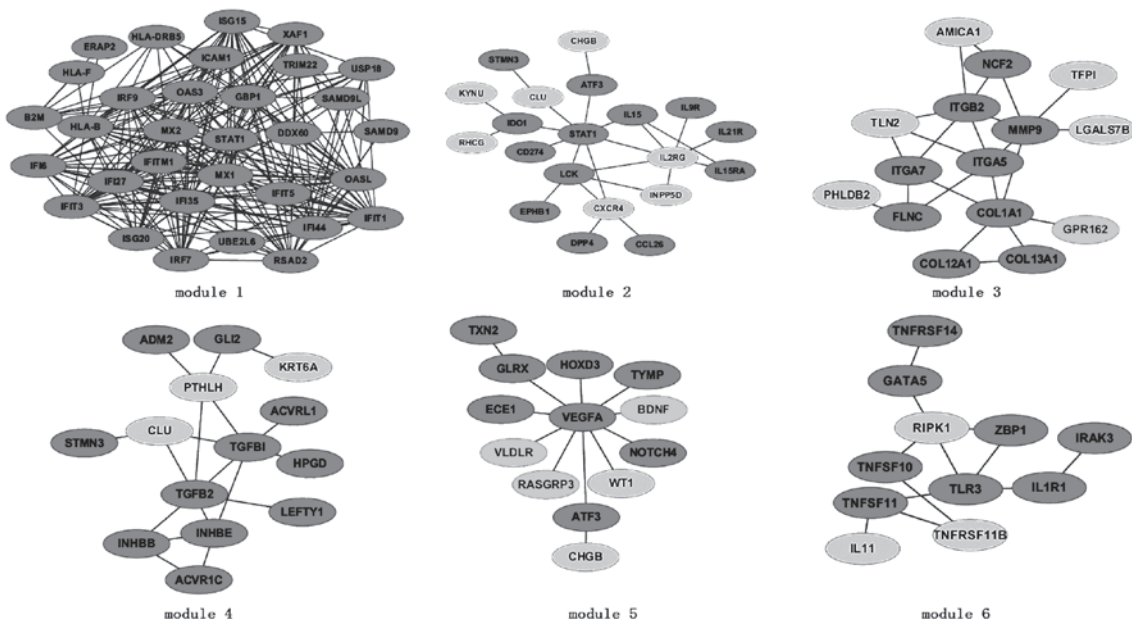


Figure 5. Six functional modules identified from the entire protein-protein interaction network using ClusterONE. Downregulated proteins in metastatic gastric cancer are in dark (n=81), upregulated proteins in gray (n=20), and non-differentially expressed ones in light gray (n=4).

autologous vaccination. A decrease in the expression of class I proteins was also reported in metastases of colorectal, gastric and laryngeal carcinomas (25). In the present study, several members of the HLA family were identified as downregulated, such as MHC class I, J (HLA-J), MHC class I, B (HLA-B) and MHC class I, F (HLA-F). Downregulation of HLA-B was also observed in metastatic serous adenocarcinomas, although the difference was not statistically significant (26).

Dedifferentiation is related to metastasis (27,28). Certain of the proteins encoded by the DEGs that are associated with this term have been reported to be linked to metastasis. Nakata *et al* (29) showed that LMO2 expression is related to aggressive behavior and distant metastasis in prostate cancer. TP63 is a suppressor of tumorigenesis and metastasis interacting with the mutant p53 protein (30). TLR3 is a member of transmembrane proteins that recognize conserved molecular motifs of viral and bacterial origin and initiate the innate immune response. Zhang *et al* (31) reported that TLR3 activation inhibits nasopharyngeal carcinoma metastasis via downregulation of the chemokine receptor CXCR4. González-Reyes *et al* (32) asserted that the expression levels of TLR3, TLR4 and TLR9 have clinical value as indicators of tumor aggressiveness in breast cancer. TWIST2 is implicated in cell lineage determination and differentiation. Fang *et al* (33) reported that TWIST2 contributes to breast cancer progression by promoting the epithelial-mesenchymal transition and cancer stem-like cell self-renewal. Similar results were observed in cervical carcinoma (34). Future studies on the proteins encoded by these DEGs may provide interesting findings and data for the development of new therapeutic targets for metastatic GC.

Six TFs were identified from the DEGs and these could regulate 169 target genes, forming 175 nodes in the transcriptional regulatory network (Fig. 3). Previous studies have shed light on their roles in the development of metastatic cancers. Ernst *et al* (35) found that STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor-mutant mice. In addition, STAT1 is also related to metastasis. Khodarev *et al* (36) point out that the STAT1 pathway mediates amplification of the metastatic potential and resistance to therapy. In cultured cell-based experiments, Greenwood *et al* (37) reported that activation of STAT1 causes increased migration and invasion, and increases the abundance of CD74. CD74 overexpression leads to increased membrane expression of proteins involved in cell adhesion and metastasis. The protein inhibitor of activated STAT1 (PIAS1) is a novel modulator of the JAK/STAT signaling pathway that negatively regulates the inflammatory response. The study by Chen *et al* (38) showed that PIAS1 is downregulated in GC tissues and involved in cell metastasis. However, according to the study by Huang *et al* (39), STAT1 is a negative regulator of tumor angiogenesis and, hence, of tumor growth and metastasis. STAT1 is an important TF regulating a range of target genes. Diverse signaling pathways dominate the physiological processes at different cellular conditions, which may explain the conflicting roles reported for STAT1 in cancer. Research on deregulated target genes of STAT1 may enhance the current understanding on the regulatory mechanisms this TF is involved in. Certain of the target genes of STAT1 have been previously associated with cancer metastasis. The gene encoding the bone

morphogenetic protein-2 (BMP-2) is one of these: Park *et al* (40) reported that BMP-2 is associated with progression to a metastatic state in GC. The study by Kang *et al* (41) further pointed out that the BMP-2 signaling pathway enhances tumor metastasis by sequential activation of the PI3K/AKT or the MAPK pathways, followed by the induction of nuclear factor- κ B and MMP-9. The chemokine C-X-C motif ligand 17 gene (*CXCL17*) is another transcriptional target of STAT1. *CXCL17* was found to be upregulated in metastatic GC in our data. Matsui *et al* (42) indicated that the *CXCL17* protein recruits immature myeloid-derived cells in tumor cells, and promotes tumor progression through angiogenesis. Future research on these target genes may reveal new therapeutic targets for metastatic GC.

EGR2 is a transcription factor with three tandem C2H2-type zinc fingers. It has been identified as a tumor suppressor, and its expression level is decreased in various types of cancer (43,44). miR-150 promotes GC proliferation by negatively regulating the pro-apoptotic gene *EGR2* (45). LaTulippe *et al* (46) reported that *EGR2* and *EGR3* are differentially expressed at least 3-fold between primary and metastatic prostate cancer. *ERG2* has numerous target genes, among which *TWIST2*, which was previously linked to metastasis (47). The RGM domain family member A (*RGMA*), a member of the repulsive guidance molecule family, is also a target gene of *ERG2*. *RGMA* is regarded as a key regulator of growth and aggressiveness of prostate cancer cells (48). Zhao *et al* (49) showed that decreased expression of *RGMA* by DNA methylation in colorectal cancer is related to tumor progression. In our data, *RGMA* was downregulated in metastatic GC. We therefore hypothesize that this gene may play a role in metastasis of GC cells.

Overall, the present study described the differential expression profiles between metastatic and non-metastatic GC. The results of this study provided meaningful data for future investigations; the identified DEGs will be useful in future research aiming to elucidate the regulatory mechanism underlying metastasis. Among these DEGs, *STAT1* and *EGR2* are promising candidates for use as metastatic GC biomarkers, and thus warrant further investigation.

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