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Original Article

Gene expression signature associated with metastasis of stomach adenocarcinoma

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Abstract: Metastasis is the leading cause of death for stomach adenocarcinoma (STAD). Molecular mechanism underlying its metastasis is still unclear. Identification of gene expression signature involved in metastasis is highly desirable. The cancer genome atlas (TCGA) data was used to obtain the mRNA expression profiles for metastatic and non-metastatic STAD samples, respectively. The differentially expressed genes (DEGs) were identified and the DEGs specifically appeared in metastatic STAD were further screened. Functional enrichment and PPI network of the DEGs were performed to determine their significance in STAD metastasis. Totally, 21 metastatic and 11 non-metastatic STAD samples were obtained, which have tumor and adjacent normal tissue in each sample. Integrated analysis led to 2979 DEGs in metastatic STAD and 491 DEGs in non-metastatic STAD with FDR<0.001. And 2593 DEGs specifically appeared in metastatic STAD. Functional annotation indicated that cell cycle was a significantly enriched GO term and KEGG pathway, which may play the important role in STAD metastasis. In metastatic STAD, HSPD1 and FHOD1 were increased, while RPS6KA6, ESRRG, BMP3 and RGMB were decreased. These DEGs may be involved in STAD metastasis process and serve as the promising metastasis biomarkers for STAD. We found specific gene expression patterns and metastasis-driving genes associated with STAD metastasis process. Our data enhances the understanding on the mechanisms underlying the STAD metastasis.

Keywords: Stomach adenocarcinoma, metastasis, biomarkers

Introduction

Stomach cancer is in fourth place behind cancers of the lung, breast, and colon and rectum. Stomach cancer is the second leading cause of cancer-related mortality rate. Almost about 42% of the cases occur in China [1]. Of the stomach cancer types, stomach adenocarcinoma (STAD) accounts for about 90% of the cases, which comprises intestinal and diffuse types [2].

Many patients with STAD are diagnosed at advanced stage with metastasis, leading to poor prognosis [3]. The presence of lymph node metastasis can decrease the survival rates of patients considerably [4]. Even after curative resection, 50-60% of patients relapse locally or with distant metastasis [5], and the metastatic disease are not curable. This situation is mainly caused by lack of satisfactory diagnostic assays for early detection and poorly understood mechanisms of tumor metastasis. Thus, metastasis is considered as an essential event in the prognosis of STAD, which reflects the disease situation and contributes to accurate prognosis.

Metastasis represents a multi-step cell-biological process, along with the cancer cells spreading to other organ sites and their subsequent adaptation to foreign microenvironments [6]. Firstly, the cancer cells escape from primary site through extracellular matrix (ECM). Then the cancer cells enter into the lumina of lymphatic or blood vessels and they can disseminate widely through circulation. Once the circulating tumor cells (CTCs) travel to the secondary loci, CTCs extravasate and survive in the foreign microenvironment. Finally, they generate clinically detectable neoplastic growths [7]. Taken together, the metastasis is formed with a complex succession of invasion-metastasis cascade, which involves a bunch of gene alterations. Discovering metastasis biomarkers or gene signatures could provide more compre-

Table 1. STAD samples used in this study

Sample type	Sample ID			
STAD without metastasis	TCGA.BR.6457	TCGA.BR.6852	TCGA.BR.7704	TCGA.BR.7715
	TCGA.CG.5720	TCGA.FP.7735	TCGA.HU.A4GH	TCGA.IN.AB1V
	TCGA.BR.6454	TCGA.BR.7851	TCGA.IN.AB1X	
STAD with metastasis	TCGA.BR.6458	TCGA.BR.6564	TCGA.BR.6802	TCGA.BR.7716
	TCGA.CG.5722	TCGA.CG.5734	TCGA.FP.7829	TCGA.HU.8238
	TCGA.HU.A4GN	TCGA.HU.A4GP	TCGA.HU.A4GY	TCGA.HU.A4HB
	TCGA.IN.8663	TCGA.IP.7968	TCGA.BR.6453	TCGA.CG.5721
	TCGA.IN.8462	TCGA.BR.7717	TCGA.HU.A4G3	TCGA.IN.7806
	TCGA.HU.A4GC			

hensive information for understanding the metastasis mechanisms, which could improve the diagnosis or prognosis of STAD.

In the present study, the mRNA expression data for metastasis and non-metastasis STAD were downloaded from The Cancer Genome Atlas (TCGA) database. The gene expression signature specifically appeared in metastasis group was identified. Moreover, their potential biological functions of the differentially expressed genes (DEGs) were further discovered to understand the molecular mechanisms of STAD metastasis. Our findings will be helpful to develop efficient treatments for the disease.

Materials and methods

Data preparation

The mRNA expression data for 450 STAD samples from distinct patients were collected from the TCGA online database (http://tcga-data.nci.nih.gov), including 415 primary solid tumors and 35 normal solid tissues. Moreover, the corresponding clinical information for the patients was downloaded. Only the samples with tumor and adjacent normal tissue were retained for subsequent analysis. Accordingly, the mRNA expression data were classified into two groups, STAD with metastasis and STAD without metastasis.

Identification of differentially expressed genes

The gene expression levels (level 3) were obtained from RNA-sequencing data. To compare different conditions, the R package DESeq2 [8] was used to determine the differentially expressed genes between the tumor and normal tissues in metastasis group (metastasis *vs.* adjacent normal) and non-metastasis group (non-metastasis *vs.* adjacent normal), respectively. In this package, principle component analysis (PCA) was used to screen outliers from the libraries. *P*-value was obtained and the false discovery rate (FDR) was calculated using the Benjamini & Hockberg method. FDR<0.001 was used as the statistical cutoff for DEGs.

After the background correction according to the adjacent normal, the DEGs were further screened for the subsequent analysis, which specifically appeared in metastasis group but not in non-metastasis group.

Functional annotation

The online software GeneCodis was used to uncover the biological functions of DEGs [9]. There are three categories (biological process, molecular function and cellular component) for Gene Ontology (GO) classification, which provided a framework for the model of biology of DEGs [10]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed to detect the potential pathways for DEGs [11]. FDR<0.05 was set as the cutoff criterion for selecting significantly enriched functional GO terms and KEGG pathways.

Construction of PPI network

The Biological General Repository for Interaction Datasets (BioGRID) is an interaction repository of protein-protein interactions, genetic interactions, chemical interactions, and post-translational modifications. BioGRID was used for the analysis of the protein-protein interactions. The PPI network was then visualized using Cytoscape software [12].

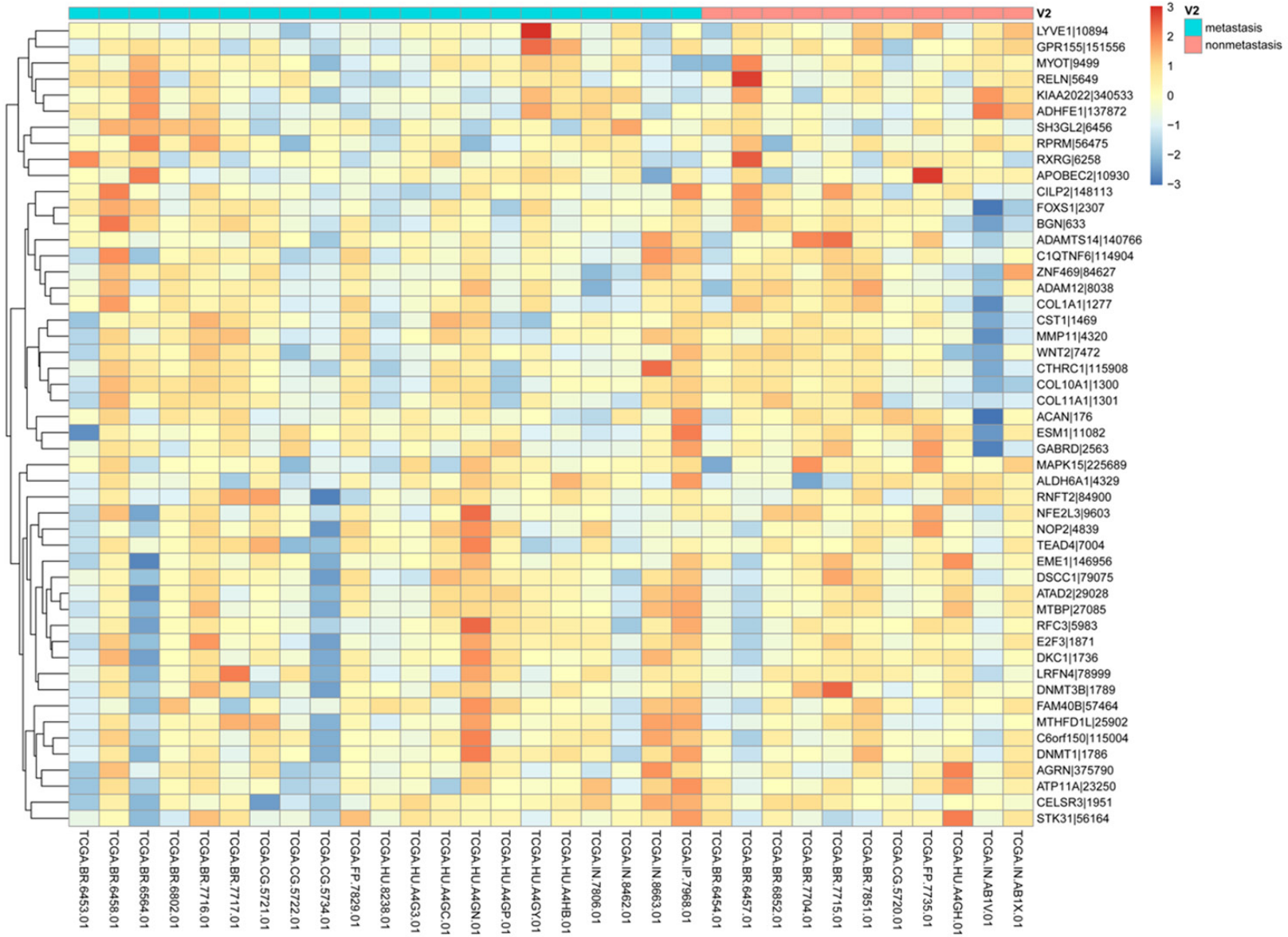

Figure 1. Heatmap of the top fifty DEGs appearing in both non-metastasis and metastasis groups. Metastasis indicates STAD metastasis group (21 samples with tumor and adjacent normal tissue); Nonmetastasis indicates STAD non-metastasis group (11 samples with tumor and adjacent normal tissue).

Table 2. Top ten up-regulated and down-regulated DEGs specifically appeared in metastasis group of STAD

Genes	LogFC	*P*-value	FDR	Genes	LogFC	*P*-value	FDR
Up-regulated				Down-regulated			
QSOX2	1.34313	1.72E-26	4.73E-23	ACADL	-3.589638	6.41E-23	9.62E-20
PUS1	1.23323	1.06E-19	1.03E-16	ROGDI	-1.390518	4.19E-18	2.77E-15
BAAT	4.52885	6.14E-19	5.33E-16	BMP3	-5.014187	1.15E-17	7.32E-15
TREM2	3.41436	1.34E-17	8.20E-15	CADM2	-4.325526	3.14E-17	1.85E-14
ODF2	0.98275	4.00E-16	1.69E-13	ESRRG	-3.838979	1.20E-16	5.80E-14
SP140L	1.20351	1.00E-15	3.59E-13	CAB39L	-2.632574	1.62E-16	7.63E-14
LOC286467	3.83483	1.32E-15	4.44E-13	RPS6KA6	-3.532646	3.08E-16	1.34E-13
FANCB	1.87519	4.37E-15	1.20E-12	GPM6B	-3.420741	1.38E-15	4.57E-13
HSPD1	1.08987	4.82E-15	1.30E-12	RGMB	-2.183304	2.14E-15	6.68E-13
FHOD1	1.03704	5.15E-15	1.37E-12	CKMT2	-3.858545	3.03E-15	8.82E-13

Online-validation of DEGs

Gene Expression Omnibus (GEO) is a public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/). Two mRNA expression datasets of STAD were downloaded from GEO database, with tumors and the normal tissues in each datasets. There were 38 cases and 31 controls in GSE13911, while there were 12 cases and 12 controls in GSE19826. According to their expression level in different STAD samples and normal tissues, a boxplot was then drawn to show the expression of differentially expressed genes in our present study using the R package.

Results

Gene expression signatures in non-metastasis and metastasis groups of STAD

Based on clinical information in the TCGA stomach adenocarcinoma, the mRNA expression data were classified into two groups, STAD with metastasis and STAD without metastasis. There were 11 STAD samples without metastasis and 21 STAD samples with lymph node metastasis or distant metastasis, respectively. TCGA barcode IDs for each sample were listed in **Table 1**.

After downloading the gene expression data, differential expression analysis was performed to identify the DEGs in non-metastasis group and metastasis group of STAD, respectively. Totally, 491 DEGs were identified in non-metastasis group with FDR<0.001, including 247 up-regulated DEGs and 244 down-regulated DEGs. Moreover, There were 2979 DEGs in metastasis group with FDR<0.001, including 1251 up-regulated DEGs and 1728 down-regulated DEGs.

We found that there were 386 DEGs appearing in both non-metastasis and metastasis groups, which may be involved in the STAD tumorigenesis. A heatmap was then generated based on the top fifty DEGs, which showed that their expression patterns were similar in non-metastasis group and metastasis group (**Figure 1**).

DEGs correlated to metastasis of STAD

After the background correction according to the adjacent normal, the DEGs specifically appeared in metastasis group were further screened out. We first excluded the DEGs in non-metastasis group, and a total of 2593 DEGs were obtained, which may be more closely associated with the STAD metastasis process. Top ten up-regulated and down-regulated DEGs specifically appeared in metastasis group of STAD were listed in **Table 2**.

GO and KEGG enrichment analysis

To gain insight into the biological function of the 2593 DEGs, we performed GO classification and KEGG pathway enrichment analysis. We found that mitotic cell cycle (GO: 0000278, FDR = 1.27E-26), cell division (GO: 0051301, FDR = 1.36E-24), cell cycle (GO: 0007049, FDR = 7.44E-22), signal transduction (GO: 0007165, FDR = 8.38E-21) and DNA replication (GO: 0006260, FDR = 1.67E-20) were significantly enriched terms for biological process. While the

Table 3. Top 15 enriched GO terms for DEGs specifically appeared in metastasis group of STAD

Items	Items_Details	Count	FDR
Biological process			
GO: 0000278	Mitotic cell cycle	87	1.27E-26
GO: 0051301	Cell division	81	1.36E-24
GO: 0007049	Cell cycle	98	7.44E-22
GO: 0007165	Signal transduction	182	8.38E-21
GO: 0006260	DNA replication	53	1.67E-20
GO: 0006355	Regulation of transcription, DNA-dependent	221	4.95E-19
GO: 0007067	Mitosis	56	3.03E-18
GO: 0000087	M phase of mitotic cell cycle	39	7.38E-18
GO: 0000236	Mitotic prometaphase	32	3.49E-13
GO: 0006281	DNA repair	59	2.14E-11
GO: 0008285	Negative regulation of cell proliferation	66	2.30E-11
GO: 0007155	Cell adhesion	91	2.43E-11
GO: 0006271	DNA strand elongation involved in DNA replication	18	2.47E-11
GO: 0006936	Muscle contraction	31	8.06E-11
GO: 0007411	Axon guidance	58	1.65E-09
Molecular function			
GO: 0005515	Protein binding	683	7.48E-91
GO: 0000166	Nucleotide binding	360	2.46E-54
GO: 0046872	Metal ion binding	395	9.30E-37
GO: 0005524	ATP binding	252	1.21E-36
GO: 0003677	DNA binding	259	1.00E-26
GO: 0008270	Zinc ion binding	261	4.98E-22
GO: 0016787	Hydrolase activity	159	4.06E-21
GO: 0042803	Protein homodimerization activity	86	1.54E-11
GO: 0003700	Sequence-specific DNA binding transcription factor activity	124	1.11E-10
GO: 0005509	Calcium ion binding	99	2.04E-10
GO: 0003676	Nucleic acid binding	114	2.07E-10
GO: 0003779	Actin binding	52	5.63E-08
GO: 0042802	Identical protein binding	53	6.22E-08
GO: 0003723	RNA binding	87	1.10E-07
GO: 0003924	GTPase activity	44	1.53E-07
Cellular component			
GO: 0005634	Nucleus	838	2.60E-119
GO: 0005737	Cytoplasm	781	6.50E-99
GO: 0005829	Cytosol	330	2.96E-40
GO: 0005886	Plasma membrane	468	2.08E-39
GO: 0005730	Nucleolus	247	2.53E-36
GO: 0016020	Membrane	500	2.95E-35
GO: 0016021	Integral to membrane	507	1.42E-28
GO: 0005654	Nucleoplasm	163	1.65E-27
GO: 0005856	Cytoskeleton	157	3.25E-27
GO: 0005739	Mitochondrion	198	1.78E-19
GO: 0005622	Intracellular	246	3.46E-17
GO: 0005694	Chromosome	64	1.18E-16
GO: 0000776	Kinetochore	25	3.12E-13
GO: 0030054	Cell junction	84	3.33E-13
GO: 0005887	Integral to plasma membrane	141	5.38E-13

Table 4. Top 15 enriched KEGG pathways for DEGs specifically appeared in metastasis group of STAD

Items	Items_Details	Count	*P*-value	FDR
hsa04270	Vascular smooth muscle contraction	32	7.10E-12	1.55E-09
hsa03030	DNA replication	17	3.70E-11	2.69E-09
hsa04020	Calcium signaling pathway	40	3.13E-11	3.41E-09
hsa04110	Cell cycle	32	8.22E-11	4.48E-09
hsa00230	Purine metabolism	35	1.28E-09	5.57E-08
hsa04914	Progesterone-mediated oocyte maturation	24	4.00E-09	1.45E-07
hsa04114	Oocyte meiosis	27	9.41E-09	2.93E-07
hsa04010	MAPK signaling pathway	46	1.10E-08	2.99E-07
hsa04724	Glutamatergic synapse	28	3.57E-08	8.66E-07
hsa03008	Ribosome biogenesis in eukaryotes	19	5.37E-07	1.17E-05
hsa00280	Valine, leucine and isoleucine degradation	14	1.20E-06	2.38E-05
hsa05200	Pathways in cancer	47	2.52E-06	4.22E-05
hsa05412	Arrhythmogenic right ventricular cardiomyopathy	18	2.49E-06	4.52E-05
hsa04912	GnRH signaling pathway	21	4.29E-06	6.68E-05
hsa03440	Homologous recombination	10	5.90E-06	8.58E-05

significantly enriched terms for molecular function were protein binding (GO: 0005515, FDR = 7.48E-91), nucleotide binding (GO: 0000166, FDR = 2.46E-54) and metal ion binding (GO: 0046872, FDR = 9.30E-37) (**Table 3**).

Moreover, the DEGs specifically appeared in metastasis group were significantly enriched in the following four KEGG pathways, such as vascular smooth muscle contraction (FDR = 1.55E-09), DNA replication (FDR = 2.69E-09), calcium signaling pathway (FDR = 3.41E-09) and cell cycle (FDR = 4.48E-09) (**Table 4**).

PPI network construction

Based on the BioGRID database, the top 20 DEGs were used to construct the PPI network. There were 356 nodes and 358 edges in the PPI network. The three hub proteins with highest degree were HSPD1 (degree = 119), RPS6KA6 (degree = 29) and ESRRG (degree = 26) (**Figure 2**).

Online-validation of DEGs

Nine genes were randomly selected from the top 20 DEGs. The online validation revealed that their expression patterns were similar with the integrated analysis. The results showed that, ROGDI, ESRRG, RPS6KA6, BMP3 and RGMB were down-regulated, while TREM2, HSPD1, FHOD1 and PUS1 were up-regulated in gastric adenocarcinoma compared with the normal gastric tissue (**Figure 3**).

Discussion

The early diagnosis and management of STAD is challenging, and the metastasis of STAD can give rise to a global poor survival rates and constitute a major public health problem. Metastasis process comprises of a series of events involving the alterations of tumor biological characteristics, such as cell proliferation and invasion. Thus, it is urgent to identify metastasis biomarkers or gene signatures for underlying the molecular mechanisms of STAD metastasis. The mRNA expression data of patients with STAD were deposited in the TCGA Data Portal and the clinical information for metastasis was also recorded, which allow us to screen the genes associated STAD metastasis by comprehensive study of gene expression data of STAD with metastasis and STAD without metastasis.

By bioinformatics analysis of 11 STAD without metastasis, a total of 491 DEGs (247 up-regulated and 244 down-regulated) were identified. There were 2979 DEGs (1251 up-regulated and 1728 down-regulated) in metastasis group (21 STAD samples). Furthermore, by comparing with the non-metastasis group, 2593 DEGs specifically appeared in metastasis group, implying that abundant of altered genes may be involved in the process of STAD metastasis. Functional annotation of the 2593 DEGs showed that cell cycle was a significantly enriched GO term and KEGG pathway, which may play important roles

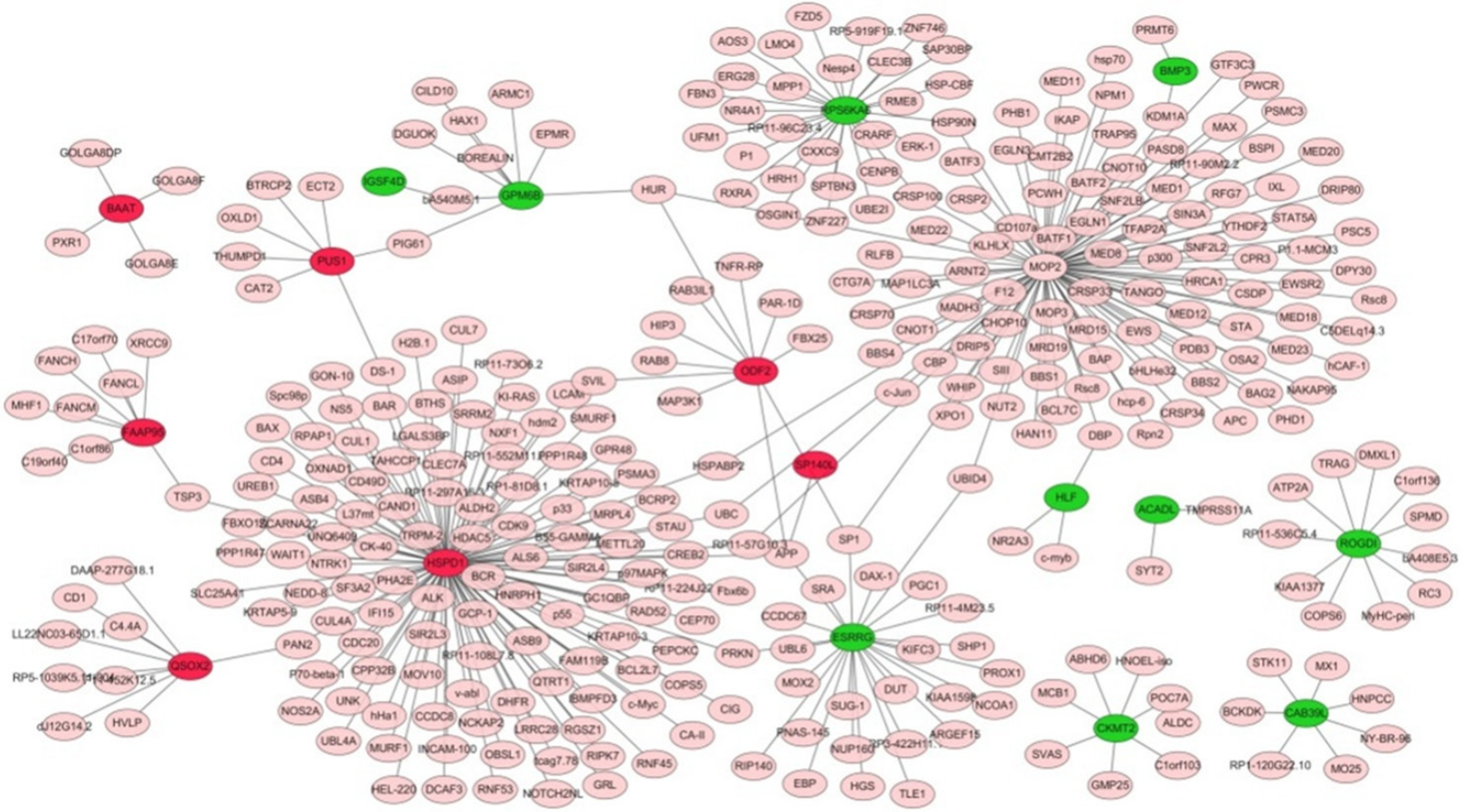

Figure 2. PPI network of the top 10 up-regulated and down-regulated DEGs. Red indicates up-regulated DEGs, and green indicates down-regulated DEGs.

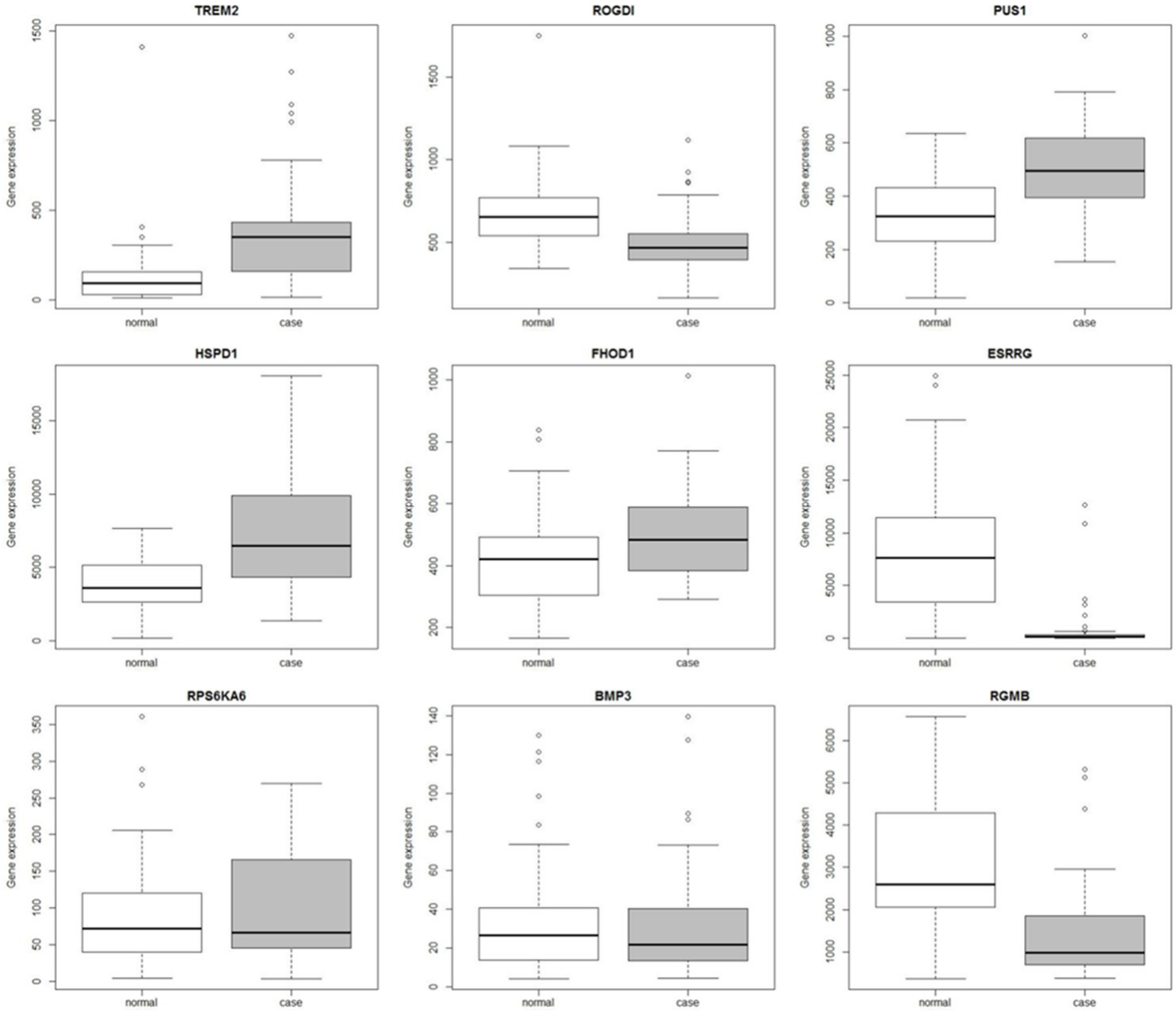
TREM2
ROGDI
PUS1
HSPD1
FHOD1
ESRRG
RPS6KA6
BMP3
RGMB
Gene expression
normal
case

Figure 3. Expression patterns of differentially expressed genes in datasets of GSE13911 and GSE19826 from GEO database. Normal: normal gastric tissue (n = 43); Case: stomach adenocarcinoma (n = 50).

in STAD metastasis. Moreover, we also found that calcium signaling pathway, MAPK signaling pathway, glutamatergic synapse and GnRH signaling pathway were enriched, which may affect the central nervous system, resulting in activation of transcription factors and rapid induction of early genes related to STAD metastasis.

To discover the critical DEGs associated to STAD metastasis, we focused on the top ten up-regulated DEGs and the top ten down-regulated DEGs. Based on them, a PPI network was established, in which HSPD1, RPS6KA6 and ESRRG were the three hub proteins with the highest degree.

HSPD1 was a member of the HSP60 gene family. Mirzaei found that HSPD1 was up-regulated in gastric adenocarcinoma cell line, and he suggested that overexpression of HSPD1 is a normal response of tumor cells to escape from apoptosis [13]. Experiments on mice model revealed that HSPD1 protein is up-regulated after Helicobacter pylori-infection, which may be involved in carcinogenesis and metastasis of gastric cancer [14]. HSPD1 was also reported to be involved in the tumor development and lymph node metastasis of lung cancer [15]. In the present study, we found that HSPD1 was a significantly up-regulated gene which specifically appeared in STAD metastasis group. Accordingly, it seemed that HSPD1 may be a promising metastasis biomarker for patients with STAD.

RPS6KA6 played a role in cell growth and proliferation. A previous study showed that RPS6KA6 was overexpressed in renal cell carcinoma compared with normal tissues, and the overexpression of RPS6KA6 could promote cell cycle progression, tumor invasion, and tumor migration in renal cell carcinoma cell lines [16]. However, the expression level of RPS6KA6 was lower in breast cancer with large tumor, high clinical stage and lymph node metastasis [17, 18]. We found that RPS6KA6 was down-regulated in STAD metastasis group, implying that it may enhance the invasive and metastatic capability of STAD. Further molecular studies will be needed to uncover the regulatory mechanism of RPS6KA6 in STAD metastasis.

ESRRG was reported as one of the five gastric cancer signature genes, which can classify the prognosis of gastric cancer [19]. Differential network analysis also revealed that ESRRG played important roles in gastric carcinogenesis [20]. Moreover, ESRRG was a target of miR-545, which was significantly correlated with histological grade and metastasis of hepatocellular carcinoma [21]. Herein, ESRRG was down-regulated in STAD metastasis group, and we speculated that ESRRG may be closely related to the metastasis process of STAD.

In addition, in the top ten up-regulated and down-regulated genes, FHOD1 may be associated with STAD metastasis. As previously reported, the miR-200c will impact on metastasis of breast cancer cells by targeting of FHOD1 [22]. Moreover, the methylation state of the BMP3 was significantly different between metastatic and non-metastatic gastric cancer samples, which may correlate with both the development and progression of gastric cancer [23, 24]. We found that BMP3 was significantly down-regulated in STAD metastasis group, which providing additional evidence for its potential roles in the STAD metastasis. Furthermore, lower levels of RGMB expression in the primary tumor correlated with local recurrences and distant metastases [25]. Previous study showed that RGMB was down-regulated in non-small cell lung cancer, which can enhance metastatic potential of NSCLC cell lines and in vivo mouse model [26]. RGMB was also a key regulator of the growth and aggressiveness of prostate cancer cells [27].

By comprehensive analysis of the gene expression data of STAD with metastasis and STAD without metastasis, a total of 2593 DEGs specifically appeared in STAD metastasis group. The three hub proteins (HSPD1, RPS6KA6 and ESRRG) in PPI network will be useful metastasis biomarker for STAD patients. The other three significantly differentially expressed genes (FHOD1, BMP3 and RGMB) may be also involved in the STAD metastasis process. These advances provide some new insights for future research on metastasis.

Disclosure of conflict of interest

None.

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