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ORIGINAL ARTICLE

Key Genes in Stomach Adenocarcinoma Identified via Network Analysis of RNA-Seq Data

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Abstract RNA-seq data of stomach adenocarcinoma (STAD) were analyzed to identify critical genes in STAD. Meanwhile, relevant small molecule drugs, transcription factors (TFs) and microRNAs (miRNAs) were also investigated. Gene expression data of STAD were downloaded from The Cancer Genome Atlas (TCGA). Differential analysis was performed with package *edgeR*. Relationships with correlation coefficient > 0.6 were retained in the gene co-expression network. Functional enrichment analysis was performed for the genes in the network with DAVID and KOBASS 2.0. Modules were identified using Cytoscape. Relevant small molecules drugs, transcription factors (TFs) and microRNAs (miRNAs) were revealed by using CMAP and WebGestalt databases. A total of 520 DEGs were identified between 285 STAD samples and 33 normal controls, including 244 up-regulated and 276 down-regulated genes. A gene co-expression network containing 53 DEGs and 338 edges was constructed, the genes of which were significantly enriched in focal adhesion, ECM-receptor interaction and vascular smooth muscle contraction pathways. Three modules were identified from the gene co-expression network and they were associated with skeletal system development, inflammatory response and positive regulation of cellular process, respectively. A total of 20 drugs, 9 TFs and 6 miRNAs were acquired that may regulate the DEGs. NFAT-COL1A1/ANXA1, HSF2-FOS, SREBP-IL1RN and miR-26-COL5A2 regulation axes may be important mechanisms for STAD.

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Keywords Stomach adenocarcinoma · Gene expression data · Differentially expressed genes · Gene co-expression network · Functional enrichment analysis

Introduction

Stomach adenocarcinoma (STAD) accounts for 90% of stomach cancer, which is the fifth most common cancer worldwide. *Helicobacter pylori* (Hp) infection is an established risk factor for stomach cancer, while tobacco smoking and dietary factors are also involved in the genesis of stomach cancer [1]. Though the incidence of stomach cancer is decreasing due to improvements in socioeconomic conditions and living habit, the prognosis of stomach cancer is generally poor, with an average 5-year survival rate of less than 10% [2]. Thus, exploration of its underlying pathogenesis to develop effective therapeutic strategies is the main aim recently.

Several molecular alterations have been identified in the development of STAD. For example, Her3 expression is shown to be significantly increased in stomach cancer compared with adjacent normal stomach tissues, leading to poor prognosis [3]. Further mechanism analysis confirms Her3 may promote cell migration and metastasis by down-regulating matrix metalloproteinases (MMPs) via PI3K/AKT signaling [4]. Zhang et al. report that reduced SIRT1 may be associated with the progression of STAD. Silencing of SIRT1 increases cell proliferation, accelerates the G1-S phase transition and reduces apoptosis [5]. Besides, increasing evidence suggests the transcription factors (TFs) and microRNAs (miRNAs) play critical roles in tumorigenesis by regulating their target genes. As an example, overexpression of transcription factor forkhead box M1 (FOXM1) has been demonstrated to be a significant, independent prognostic factor for survival of patients with stomach cancer [6]. Lactate dehydrogenase A (LDHA) is transcriptionally activated

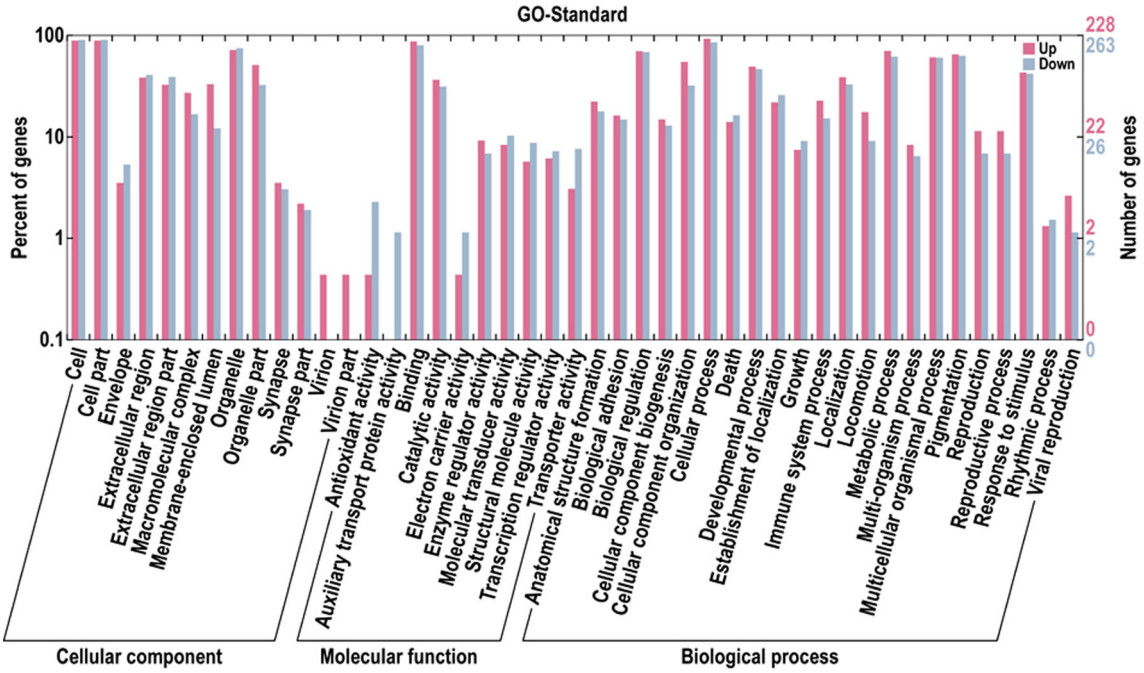

Fig. 1 GO annotations for the differentially expressed genes. Up-regulated genes are in red while down-regulated genes are in gray. Left vertical axis indicates percentage of genes while right vertical axis indicates number of genes

by FOXM1 to promote glycolysis and progression of stomach cancer [7]. Zhang et al. identify both miR-107 and miR-25 can promote proliferation and invasion of STAD cells by targeting large tumor suppressor 2 (LATS2) [8]. However, exact mechanisms of STAD remain unclear.

The goal of this study was to further screen important genes associated with STAD by differentially expressed genes (DEGs) analysis, network and module construction of gene expression profile data using bioinformatics tools. Besides, relevant small molecules drugs, TFs and miRNAs were disclosed, which might be beneficial for identification of therapeutic targets.

Materials and Methods

Raw Data and Pre-Treatment

Gene expression data (RNASeqV2) of STAD were downloaded from The Cancer Genome Atlas (TCGA) with TCGA-

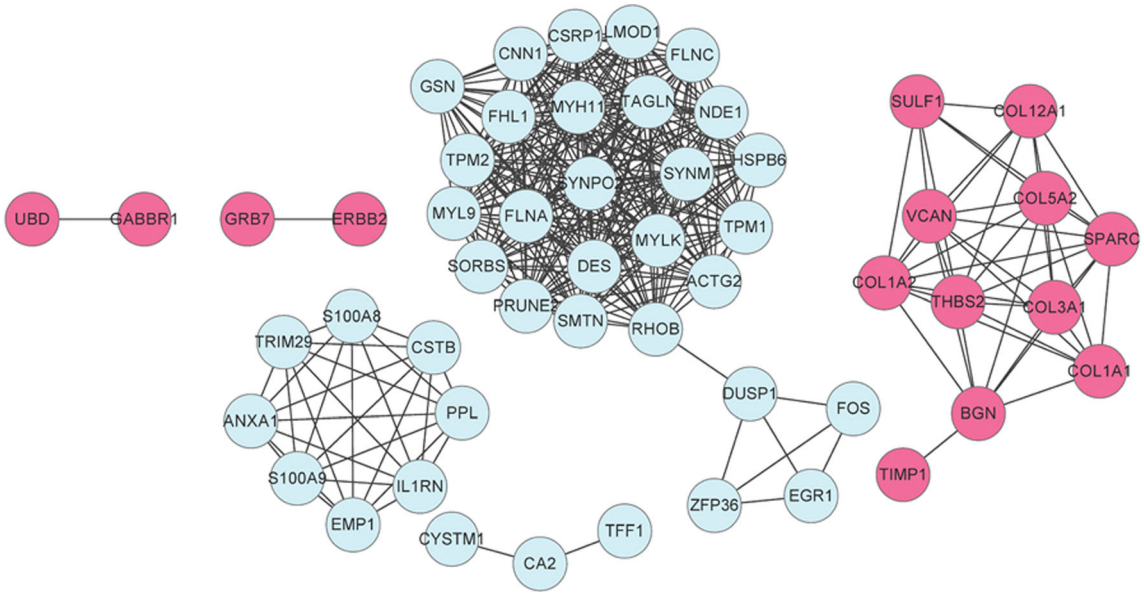

Fig. 2 The gene co-expression network of differentially expressed genes. Up-regulated genes in red, while down-regulated genes are in blue

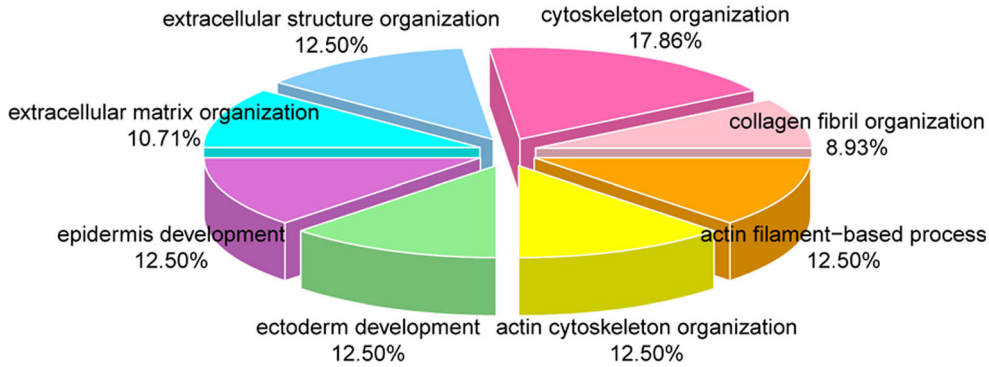

Fig. 3 GO terms significantly over-represented in the genes from the co-expression network

Aseembler, including 285 STAD samples and 33 normal controls. The gene expression level was measured as the reads per kilobase per million reads (RPKM). Raw count data were normalized with the tag count comparison (TCC) package [9].

Screening of DEGs

Differential expression analysis between STAD samples and normal controls was performed with package *edgeR* [10] of *R*. *P* value was adjusted with package *multtest* [11]. False positive rate (FDR) < 0.05 and |log2FC (fold change) | > 1 were set as the cut-off point to screen out DEGs.

Construction of a Gene Co-Expression Network

Correlation between DEGs were calculated with package *Ebcoexpress* [12]. Relationships with correlation coefficient > 0.6 were retained in the gene co-expression network that was visualized with Cytoscape [13].

Functional Enrichment Analysis

Gene Ontology (GO) annotation of all DEGs were obtained by mapping to GO database (http://www.geneontology.org/), after which the number of genes in certain functional terms was calculated with a Perl script. GO enrichment analysis was performed for the DEGs in the gene co-expression network with DAVID (Database for Annotation, Visualization and Integration Discovery, http://david.abcc.ncifcrf.gov/) [14]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also carried out for the DEGs in the gene co-expression network with KOBASS 2.0 [15]. *P* value <0.05 was set as the threshold.

Module Analysis

Modules were identified with Mcode [16] of Cytoscape [13] (degree cutoff > = 2 and k-core > = 2). Functional annotations were given for each module with Bingo [17] based on hypergeometric distribution (adjusted *p*-value <0.01).

Screening of Relevant Small Molecule Drugs, miRNAs and TFs

Relevant small molecules drugs were predicted by Connectivity map (cmap) [18] and those with |score| > 0.8 were retained. Relevant miRNAs and TFs were searched with WebGestalt [19, 20]. Adjusted *p*-value <0.05 was set as the threshold.

Results

Differentially Expressed Genes Identification and GO Annotation

A total of 12,877 genes were included in 318 samples collected from the RNA-seq data. According to the cut-off point (FDR < 0.05 and |log2FC| > 1), 520 DEGs were obtained between STAD and normal samples, including 244 up-regulated and 276 down-regulated genes.

DEGs were mapped to GO database to obtain their underlying functions. As displayed in Fig. 1, DEGs may play roles

Table 1 KEGG pathways significantly over-represented in the genes from the gene co-expression network

Term	Count	*P*-value	Genes
hsa04510:Focal adhesion	10	2.27E-08	ERBB2, COL3A1, COL1A2, COL1A1, FLNC, COL5A2, THBS2, FLNA, MYLK, MYL9
hsa04512:ECM-receptor interaction	5	2.74E-04	COL3A1, COL1A2, COL1A1, COL5A2, THBS2
hsa04270:Vascular smooth muscle contraction	4	9.02E-03	ACTG2, MYH11, MYLK, MYL9

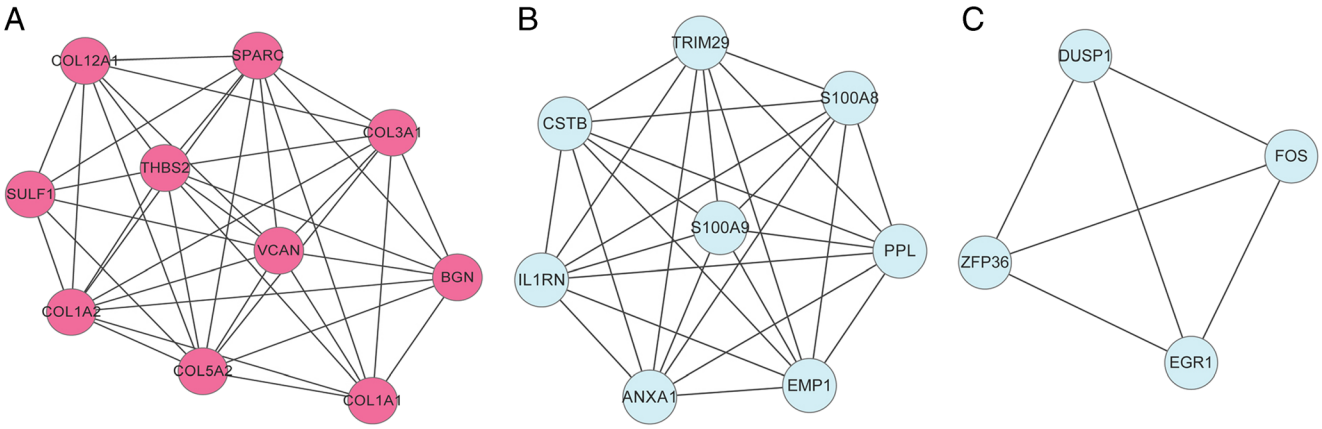

Fig. 4 Three modules identified from the gene co-expression network. Up-regulated genes are in red, while down-regulated genes are in blue

Table 2 Functional terms of three modules

Module A				
GO-ID	*P*-value	Adjusted *p*-value	Genes	Description
1501	2.90E-08	1.64E-06	COL3A1, COL1A2, COL12A1, SPARC, COL1A1, COL5A2	Skeletal system development
48,731	2.17E-05	4.55E-04	BGN, COL3A1, COL1A2, COL12A1, VCAN, SPARC, COL1A1, COL5A2	System development
48,856	4.40E-05	7.64E-04	BGN, COL3A1, COL1A2, COL12A1, VCAN, SPARC, COL1A1, COL5A2	Anatomical structure development
7275	1.03E-04	1.46E-03	BGN, COL3A1, COL1A2, COL12A1, VCAN, SPARC, COL1A1, COL5A2	Multicellular organismal development
32,502	1.95E-04	2.21E-03	BGN, COL3A1, COL1A2, COL12A1, VCAN, SPARC, COL1A1, COL5A2	Developmental process
32,501	1.83E-03	1.33E-02	BGN, COL3A1, COL1A2, COL12A1, VCAN, SPARC, COL1A1, COL5A2	Multicellular organismal process
16,043	3.57E-03	2.24E-02	COL3A1, COL1A2, COL12A1, SPARC, COL1A1, COL5A2	Cellular component organization
Module B				
GO-ID	*P*-value	Adjusted *p*-value	Genes	Description
6954	1.51E-05	2.27E-03	S100A8, IL1RN, S100A9, ANXA1	Inflammatory response
9611	1.25E-04	4.45E-03	S100A8, IL1RN, S100A9, ANXA1	Response to wounding
30,855	8.53E-05	4.45E-03	PPL, ANXA1, EMP1	Epithelial cell differentiation
8544	1.15E-04	4.45E-03	PPL, ANXA1, EMP1	Epidermis development
7398	1.47E-04	4.45E-03	PPL, ANXA1, EMP1	Ectoderm development
6952	2.13E-04	5.35E-03	S100A8, IL1RN, S100A9, ANXA1	Defense response
7626	3.59E-04	7.73E-03	S100A8, S100A9, CSTB	Locomotory behavior
Module C				
GO-ID	*P*-value	Adjusted *p*-value	Genes	Description
48,522	3.84E-04	6.20E-03	ZFP36, EGR1, FOS, DUSP1	Positive regulation of cellular process
48,518	5.66E-04	6.75E-03	ZFP36, EGR1, FOS, DUSP1	Positive regulation of biological process
80,090	3.81E-03	2.17E-02	ZFP36, EGR1, FOS, DUSP1	Regulation of primary metabolic process
31,323	4.65E-03	2.32E-02	ZFP36, EGR1, FOS, DUSP1	Regulation of cellular metabolic process
19,222	5.63E-03	2.66E-02	ZFP36, EGR1, FOS, DUSP1	Regulation of metabolic process
71,310	1.82E-05	4.99E-03	EGR1, FOS, DUSP1	Cellular response to organic substance
70,887	8.44E-05	6.20E-03	EGR1, FOS, DUSP1	Cellular response to chemical stimulus
9725	1.14E-04	6.20E-03	EGR1, FOS, DUSP1	Response to hormone stimulus
9719	1.59E-04	6.20E-03	EGR1, FOS, DUSP1	Response to endogenous stimulus

in the progression of STAD by involving in biological adhesion, death and immune system process, which all seemed to be cancer-related.

Gene Co-Expression Network Construction and Functional Enrichment Analysis

A gene co-expression network, containing 53 DEGs (38 down-regulated genes and 15 up-regulated genes) and 338 edges, was established (Fig. 2). GO enrichment analysis for the genes in co-expression network revealed 8 significantly over-represented terms, such as ectoderm development and actin cytoskeleton organization (Fig. 3). KEGG pathway enrichment analysis also suggested that these genes (i.e. collagen type I alpha 1 chain, COL1A1; collagen type V alpha 2 chain, COL5A2) may be related with focal adhesion, ECM-receptor interaction and vascular smooth muscle contraction pathways (Table 1).

Modules Analysis and Function Enrichment Analysis

To further screen important genes associated with STAD, module analysis was performed using the genes identified from the gene co-expression network. As a result, 3 modules were obtained (Fig. 4), among which module A included 10 genes, such as COL1A1 and COL5A2; module B contained 8 genes, such as interleukin 1 receptor antagonist (IL1RN) and annexin A1 (ANXA1); and module C included 4 genes, such as dual specificity phosphatase 1 (DUSP1) and FBJ murine osteosarcoma viral oncogene homolog (FOS). Functional enrichment analysis indicated that skeletal system development, inflammatory response and positive regulation of cellular process were significant in these 3 modules, respectively (Table 2).

Relevant Small Molecule Drugs, miRNAs and TFs

A total of 20 relevant small molecule drugs were identified (Table 3). Camptothecin and Menadione had the maximum negative correlation coefficient, indicating their underlying therapeutic effects for STAD.

Besides, a total of 9 TFs, such as nuclear factor of activated T cells (NFAT), forkhead box J2 (FOXJ2), heat shock transcription factor 2 (HSF2), sterol-regulatory element-binding protein (SREBP), vitamin D receptor (VDR) and myeloid zinc finger 1 (MZF1), as well as 6 miRNAs, including miR-148a, miR-19, miR-200, miR-17, miR-26 and miR-506, were revealed by WebGestalt (Tables 4 and 5). These TFs and miRNAs may be involved in STAD by regulating their differential targets genes, such as NFAT-COL1A1/ANXA1, HSF2-FOS, SREBP-IL1RN and miR-26-COL5A2 (Tables 4 and 5).

Discussion

By establishing gene co-expression network and modules analysis, our present study suggests some crucial genes in the pathogenesis of STAD, such as COL1A1, COL5A2, ANXA1, FOS and IL1RN. They may be involved in ECM-receptor interaction, inflammatory and regulation of biological processes to promote the development and progression of STAD.

ECM-receptor interaction is a common mechanism associated with tumor cell invasion and metastasis, including stomach cancer [21, 22]. COL1A1 and COL5A2 belong to the collagen protein family that is an essential structural component of extracellular matrix (ECM). Thus, COL1A1 and COL5A2 may be important genes for cancer, which has been demonstrated by several studies. For example, Shi et al. validate a 3.10 ± 1.08 folds up-regulation of COL1A1 in gastric cancer than that in normal ones [23] and show COL1A1 may be a potential biomarker that can distinguish between cancer and corresponding normal tissues with area under curve of 0.806 [23]. Knock-down of Col1a1 gene expression can efficiently reduce cell migration (2.5 fold, EMT6 cells; 3-fold, 4 T1 cells), but reverse results can be obtained after exogenously supplying Col1a1 [24]. COL5A2 is also reported to be up-regulated in the colorectal carcinomas, but not expressed in normal colon [25]. However, the experimental studies on COL5A2 in STAD remain rare. In line with the study of Fischer et al. [25], we also found COL5A2 is upregulated in

Table 3 Twenty relevant small molecule drugs

Cmap name	Correlation	*P*-value
Camptothecin	-0.996	0.0000
Menadione	-0.996	0.0000
Phenoxybenzamine	-0.989	0.0000
MS-275	-0.978	0.0011
Irinotecan	-0.975	0.0000
Apigenin	-0.847	0.0010
Ciclopirox	-0.831	0.0015
Norcyclobenzaprine	-0.825	0.0018
Prestwick-559	-0.806	0.0146
Iloprost	0.831	0.0096
W-13	0.846	0.0480
Bacitracin	0.847	0.0068
Prestwick-691	0.856	0.0056
Sulmazole	0.887	0.0029
Viomycin	0.897	0.0001
Harmalol	0.908	0.0016
Vigabatrin	0.916	0.0012
Isoflupredone	0.939	0.0004
Podophyllotoxin	0.969	0.0000

Table 4 Nine relevant transcription factors

Transcription factors	ID	Parameters	Genes
hsa_TGGAAA_V$NFAT_Q4_01	DB_ID: 2437	O = 73; rawP =6.73e-12; adjP =6.42e-11	CSRP1, SPARC, GSN, FLNA, CKB, ANXA1, DUSP1, MYL9, CSTB, BTG2…
hsa_TGANTCA_V$AP1_C	DB_ID: 2402	O = 56; rawP =3.11e-11; adjP =7.37e-10	CNN1, CDC6, TRIP13, MAOB, SFRP4, TPM2, AURKA, NRCAM, RFC3, COL7A1…
hsa_V$FOXJ2_02	DB_ID: 2089	O = 38; rawP =5.39e-9; adjP =2.10e-8	COCH, EPM2A, INHBB, SPEG, LIF, POU2AF1, LRP8, STIL, GNA15, AKAP6…
hsa_V$HSF2_01	DB_ID: 1951	O = 16; rawP =8.54e-10; adjP =5.98e-09	PGM5, PITX1, THY1, KRT7, HBB, NDRG4, AKR1C3, GPM6B, FOS, CBR1…
hsa_V$TATA_01	DB_ID: 2025	O = 28; rawP =1.82e-10; adjP =1.59e-09	BUB1, CES2, FBP1, ABCG2, ACE, SPP1, CCL18, KCNMB1, VILL, FAP…
hsa_V$SREBP_Q3	DB_ID: 2261	O = 23; rawP =2.85e-07; adjP =8.29e-06	RHOB, SULF1, CLIP3, CAP2, IGJ, IL1RN, SYNM, PRUNE2, MT1E, CCDC69…
hsa_V$CDC5_01	DB_ID: 2132	O = 17; rawP =4.36e-06; adjP =2.28e-05	FAP, NCAM1, OLR1, IDO1, TPX2, DNASE1, ITGAX, XDH, SLC11A1, GRB7…
hsa_V$VDR_Q6	DB_ID: 2325	O = 13; rawP =7.42e-05; adjP =0.0001	SKA1, ZBTB7C, PMEPA1, FAM129A, PRC1, TSPYL2, TRIB3, CLDN1, KIF4A, SNX10…
hsa_V$MZF1_01	DB_ID: 1899	O = 10; rawP =0.0011; adjP =0.0140	HJURP, KIF20A, DHRS11, ATAD2, NETO2, QRSL1, ITIH5, ORC6, PBK, LIPG

O, number of genes in the gene set and also in the category; rawP, *p* value from hypergeometric test; adjP, *p* value adjusted by the multiple test adjustment

STAD (log2FC = 2.05) and further research is necessary to prove its roles.

Accumulating evidence indicates that there is a close relationship between stomach cancer and chronic inflammation [26]. High inflammation response immune cells ratios (such as neutrophil to lymphocyte ratio and derived neutrophil to lymphocyte ratio) have been demonstrated to be independent poor prognostic indicators for patients with stomach cancer [27]. The pro-inflammatory cytokines secreted by immune cells are highly expressed, but anti-inflammatory cytokines are downregulated in stomach cancer [28]. In line with these studies, we also found several genes from the co-expression network and modules analysis were associated with inflammatory response, including IL1RN and ANXA1. Interleukin 1 receptor antagonist (IL1RN) inhibits the activities of pro-inflammatory cytokines interleukin 1, alpha (IL1A) and interleukin 1, beta (IL1B), and thus they may be significantly reduced in cancer, which was proved by the study of Worst et al. [29] and our study (log2FC = −3.01). ANXA1 is a membrane-localized protein that binds and inhibits phospholipase A2 to exert anti-inflammatory activity. Yu et al. indicate that ANXA1 is a negative biomarker for STAD development and progression as its expression decreases significantly when stomach cancer progresses and metastasizes [30]. Consistent with the study of Yu et al. [30], we also found ANXA1 was downregulated in GA (log2FC = −3.06). However, further studies on these critical genes are needed to advance the understanding about the pathogenesis of STAD.

Table 5 Six relevant microRNAs

miRNA	ID	Parameters	Genes
hsa_TGCACTG, MIR-148A	DB_ID: 672	O = 38; rawP =1.18e-08; adjP =3.63e-08	MALL, CHI3L1, KIF2C, DUSP5, EPHB2, MXRA5, PDLIM3, BUB1, CES2, FBP1…
hsa_TTTGCAC, MIR-19	DB_ID: 696	O = 26; rawP =8.82e-08; adjP =5.47e-07	PRUNE2, MT1E, CCDC69, CBX7, PDZRN3, KIAA1199, NCAPH, COL4A5, OLFML2B, TTC9…
hsa_CAGTATT, MIR-200	DB_ID: 679	O = 19; rawP =8.01e-08; adjP =7.61e-07	FAM129A, PRC1, TSPYL2, TRIB3, CLDN1, KIF4A, SNX10, NDE1, CEP55, C1orf115…
hsa_GCACTTT, MIR-17	DB_ID: 665	O = 21; rawP =4.15e-05; adjP =0.0001	BARX1, ASPM, KLF8, KIF26B, MFI2, HELLS, ATAD5, KLF4, AIM1L, DEPDC1…
hsa_TACTTGA, MIR-26	DB_ID: 687	O = 15; rawP =0.0010; adjP =0.0025	PITPNM3, KIF18A, TAS2R14, ADAMTS12, GDF15, HSPB8, BRIP1, COL5A2, SYT13, RNFT2…
hsa_GTGCCTT, MIR-506	DB_ID: 712	O = 9; rawP =0.0096; adjP =0.0107	DAK, LEPREL1, HJURP, KIF20A, DHRS11, ATAD2, NETO2, QRSL1, ITIH5

O, number of genes in the gene set and also in the category; rawP, *p* value from hypergeometric test; adjP, *p* value adjusted by the multiple test adjustment

FOS was initially described as an oncogene, but is found to be downregulated in cancer and associated with favorable prognosis recently [31]. Stable transfection of FOS can strongly delay tumor growth, reduce lung metastases and circulating tumor cells [32]. In our study, FOS was also lowly expressed in STAD compared with normal controls (log2FC = −1.63), indicating its important roles in STAD.

In addition, TFs and miRNAs were also predicted to further investigate the regulation mechanisms of the above genes in GA, including NFAT-COL1A1/ANXA1, HSF2-FOS, SREBP-IL1RN and miR-26-COL5A2. Although there were few studies to experimentally confirm their regulation relationships, these TFs and miRNAs have been implicated in the development of cancer. For example, NFAT plays a critical role in the development of immune system as well as cancer [33, 34]. HSF2 is suggested to act as a suppressor gene and loss of its expression is correlated with metastasis and poor survival of prostate cancer patients [35]. SREBP-1 is a key transcriptional regulator that is involved in tumor growth and progression by alterations in the lipid metabolism [36]. MiR-26 is found to be down-regulated in gastric cancer and significantly associated with shorter overall survival. Elevated expression of miR-26 significantly suppresses cell proliferation, migration, invasion and colony formation, and induces apoptosis of stomach cancer cancer cells [37].

Potential drugs for treatment of STAD were also predicted, such as camptothecin and menadione. It has reported that camptothecin inhibits human cancer xenografts in nude mice [38] and its derivative CPT-11 has been tested in clinical studies for treatment of gastric cancer [39]. Menadione is also demonstrated to induce stomach cancer cell death [40], but the clinical studies are still needed to confirm its therapeutic effects.

In conclusion, our present study suggests some crucial genes in STAD, including NFAT-COL1A1/ANXA1, HSF2-FOS, SREBP-IL1RN and miR-26-COL5A2 and their underlying mechanisms were ECM-receptor interaction, inflammatory and regulation of biological processes. Camptothecin and menadione are potential drugs for treatment of STAD.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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