Original Article

Identification of Cancer/Testis Antigens Related to Gastric Cancer Prognosis Based on Co-Expression Network and Integrated Transcriptome Analyses

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Abstract

Background: Gastric cancer is a worldwide life-threatening cancer. The underlying cause of it is still unknown. We have noticed that some cancer/testis antigens (CTAs) are up-regulated in gastric cancer. The role of these genes in gastric cancer development is not fully understood. The main aim of the current study was to comprehensively investigate CTAs` expression and function in stomach adenocarcinoma (STAD).

Materials and Methods: A comprehensive list of CTA genes was compiled from different databases. Transcriptome profiles of STAD were downloaded from the cancer genome atlas (TCGA) database and analyzed. Differentially-expressed CTAs were identified. Pathway enrichment analysis, weighted gene correlation network analysis (WGCNA), and overall survival (OS) analysis were performed on differentially-expressed CTA genes.

Results: Pathway enrichment analysis indicates that CTA genes are involved in protein binding, ribonucleic acid processing, and reproductive tissues. WGCNA showed that six differentially-expressed CTA genes, namely Melanoma antigen gene (MAGE) family member A3, A6, A12 and chondrosarcoma associated gene (CSAG) 1, 2, and 3, were correlated. Up-regulation of MAGEA11, MAGEC3, Per ARNT SIM domain containing 1 (PASD1), placenta-specific protein 1 (PLAC1) and sperm protein associated with the nucleus X-linked family member (SPANXB1) were significantly associated with lower OS of patients.

Conclusion: MAGEA11, MAGEC3, PASD1, PLAC1, and SPANXB1 can be investigated as prognostic biomarkers in basic and clinical studies. Further functional experiments are needed to understand the exact interaction mechanisms of these genes.

Keywords: Cancer/testis antigens, gastric cancer, prognostic, transcriptome

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Introduction

Gastric cancer is one of the deadliest cancers based on the GLOBOCAN 2020.[1] In recent decades, the gastric cancer burden has globally decreased. Higher socioeconomic status reduces the *Helicobacter pylori* infection, and a decrease in high-salted food consumption and smoking will lower cancer incidence.[1,2] However, the 5-year survival rate of gastric cancer continues to be poor,[1,2] except in Japan and South Korea, where wide population screening is practiced.[3] Although endoscopy is recommended as a gold standard screening method for gastric cancer,[4] it has not been utilized in low incidence regions or resource-limited countries.[5] The first-line treatment of gastric cancer is surgery and preoperative chemotherapy, but the recurrence rate remains high.[6]

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Identifying reliable diagnostic, predictive, and prognostic tumor biomarkers assist in developing an inexpensive screening method and selecting appropriate chemotherapy regimens. Nevertheless, finding a robust, patient, and disease-specific biomarker can be challenging. Today, carcinoembryonic and carbohydrate antigens 19-9 are the clinically used biomarkers for gastric cancer. However, these biomarkers are not cancer-specific.[7,8]

Next-generation sequencing technologies have provided the opportunities to study transcriptome profiling of tumors and identify differentially-expressed genes (DEGs).[9] The cancer DEGs are potential biomarkers that might participate in tumor development.

In the current study, we acquired The Cancer Genome Atlas (TCGA) TCGA-stomach adenocarcinoma (TCGA-STAD) data from the genomic data commons data portal. The gene expression profiles of the samples were analyzed, and DEGs were obtained. We noticed that four melanoma antigen gene (MAGE) family members A, which belong to cancer/testis antigens (CTAs), are among the top up-regulated genes. The expression of CTAs is restricted to the testes, trophoblast, and many tumors.[10] Due to this distinctive expression pattern of CTAs in normal tissues and tumors, these genes may be utilized as potential tumor biomarkers. Thus, we prepared a list of differentially-expressed gastric cancer CTAs. In the next step, weighted gene correlation network analysis (WGCNA) was exploited to construct a co-expression network and evaluate CTA genes interaction and pathways.

Materials and Methods

The Cancer Genome Atlas data processing

Level 3 transcriptome profiles of 443 TCGA-STAD were retrieved from the TCGA data portal (https://portal.gdc.cancer.gov) on October 19, 2020. The HTseq-counts data were downloaded, preprocessed, and normalized by R/Bioconductor package TCGABiolinks v 2.18.0.[11] The comparison between 375 tumors and 32 adjacent normal tissues allowed the identification of DEGs using the EdgeR package of TCGABiolinks and "Fisher's exact test" method. The cut-off criteria were log fold change >2.0, $P < 0.05$, and the false discovery rate -adjusted $P < 0.01$.

Cancer/testis antigen gene list preparing

Since, to the best of our knowledge, there is not any available comprehensive list of CTA genes, we searched the "cancer/testis antigen" as keywords in Gene cards, NCBI-Gene, and human genome nomenclature committee (HGNC) databases and acquired the list of CTA genes.[12-14] After searching for the "cancer/testis antigen" keyword in the NCBI-Gene database, results were filtered by "Homo sapiens" and RefSeqGene criteria. In the HGNC database, at the first step, only approved genes were selected. Then, all the gene locus types were selected except the genes with unknown locus. Moreover, the list of genes of the CTdatabase was downloaded.[15] The Venn diagram was utilized to obtain the shared genes. The Venn diagram in Figure 1 was drawn online (https://bioinformatics.psb.ugent.be/webtools/Venn/). Each area in the Venn diagram represents the set of genes in one of the databases. The number of shared genes among the databases is written at the intersection of the corresponding areas [Figure 1]. The final CTA gene list (280 genes) is represented.

Co-expression network analysis

The voom normalized gene counts were exploited to construct a co-expression network of 375 tumor samples using WGCNA, v. 1.70-3 package.[16,17] The similarity matrix was built based on the biweight midcorrelation coefficient between all genes. Subsequently, the similarity matrix was converted to an adjacency matrix by the soft threshold power of $\beta=5$. The RCy3, V.2.10.2 package was utilized to connect WGCNA to Cytoscape, V.3.8.1, and visualize the network.[18]

Pathway enrichment analysis

The g: Profiler[19] was utilized to perform pathway enrichment analysis of the differentially-expressed CTAs. The corresponding biological processes (BP), cellular components (CC), and molecular functions (MF) were identified using Gene Ontology (GO). The signaling pathways involved were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG).[20] The results were visualized by the Enrichment Map and Auto Annotate[21,22] apps of Cytoscape.

Survival analysis

The survival analysis was performed using the Kaplan–Meier curve, drawn using GraphPad Prism v. 8.4.3. The Kaplan-Meier survival curves of each gene were plotted. The $P < 0.05$ was considered statistically significant. These analyses depicted

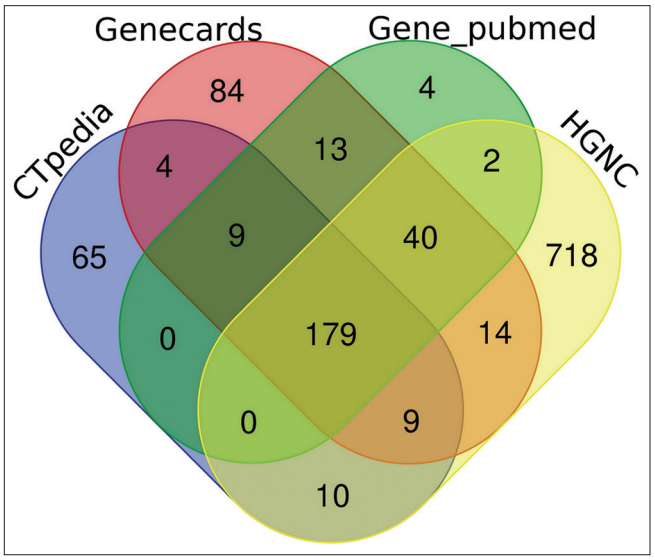

Figure 1: The Venn diagram. Blue, red, green, and yellow closed curves represent CTpedia, Genecards, Gene_pubmed, and HUGO Gene Nomenclature Committee databases, respectively. Different numbers of genes are categorized as cancer/testis antigens in databases

the relationship between differentially-expressed CTAs and overall patient survival.

Results

Differentially-expressed genes of stomach adenocarcinoma cases

The workflow for the integrated bioinformatics analysis is shown in Figure 2. The ribonucleic acid (RNA) sequencing data of TCGA-STAD were downloaded from TCGA, normalized, and filtered the outliers. The total number of genes was 20852. Gene expression profiles of tumor and adjacent normal tissue samples were compared, and 1839 DEGs were identified based on the aforementioned cut-off criteria. The up-regulated genes accounted for 55.4% (1019/1839) of all genes. Most of the top ten up-regulated DEGs were CTA genes [Table 1]. The collected CTA gene list was compared with the DEGs, and the differentially-expressed CTA genes between the tumor and adjacent normal tissues were selected. The total number of differentially-expressed CTAs was 83, including 81 coding and two noncoding RNAs.

Pathway enrichment analysis

A pathway enrichment analysis of differentially expressed CTAs (up-and down-regulated), including GO: BP, CC and MF, and KEGG pathway analysis, was conducted. GO functions were mainly enriched in protein binding, RNA processing, and reproductive process [Figure 3]. Some of the expressed

Table 1: Top ten up-regulated and differentially-expressed genes in stomach adenocarcinoma tissues of The Cancer Genome Atlas

Gene	logFC	*P*	CTAs/others
MAGEA12	9.1	3E-82	CTA
MAGEA4	8.9	6E-84	CTA
MAGEA6	8.8	4E-81	CTA
MAGEA3	8.8	8E-82	CTA
HDGFL1	8.2	7E-62	Others
FGF19	8.2	3E-72	Others
VCX2	8.1	2E-56	CTA
APOA2	8.1	1E-75	Others
MAGEA1	7.9	4E-69	CTA
HOXC12	7.9	5E-71	Others

Transcriptome data analysis of gastric cancer from the TCGA database identified 1839 DEGs. CTAs consist of 79 up-regulated and four down-regulated genes. The MAGEA gene family members are the most up-regulated genes in TCGA-STAD compared to adjacent normal tissues. MAGEA: Melanoma antigen family A, TCGA: The cancer genome atlas, DEG: Differentially-expressed gene, TCGA-STAD: TCGA-stomach adenocarcinoma, CTA: Cancer/testis antigens, FC: Fold change

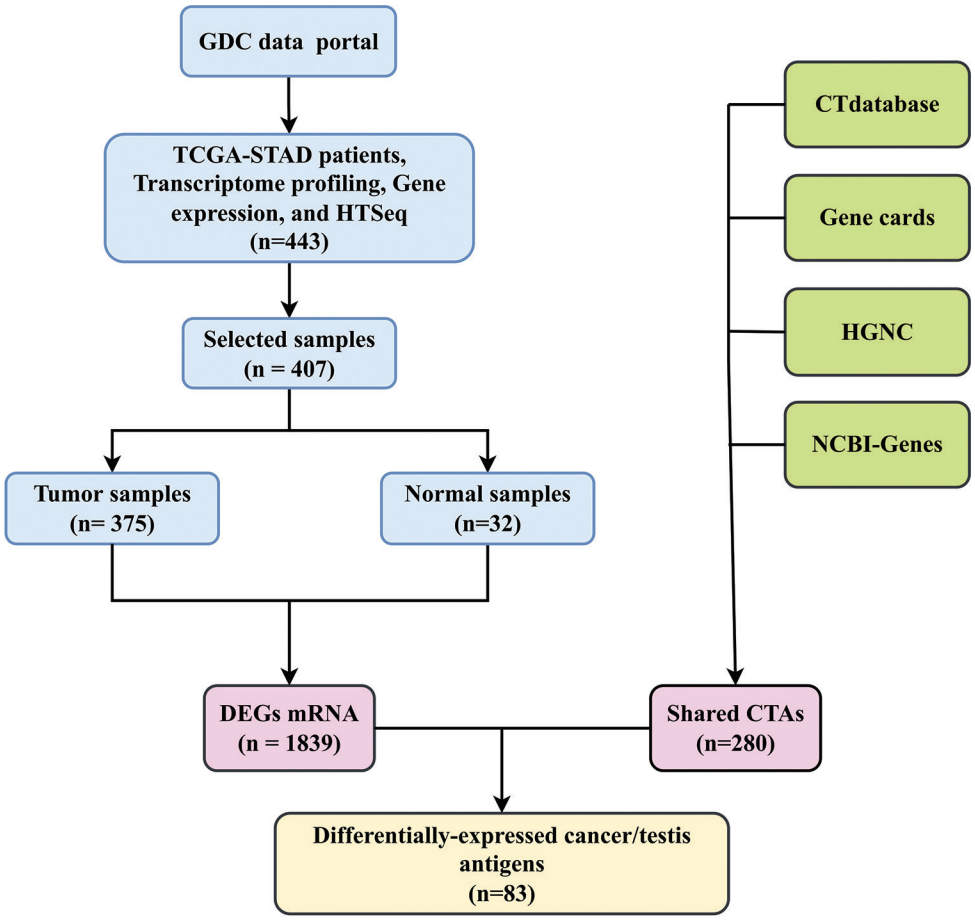

Figure 2: Flow chart of the current study. Gene expression analysis pipeline of 443 gastric cancer patients is depicted on the left. Preparing the list of cancer/testis antigens is shown on the right

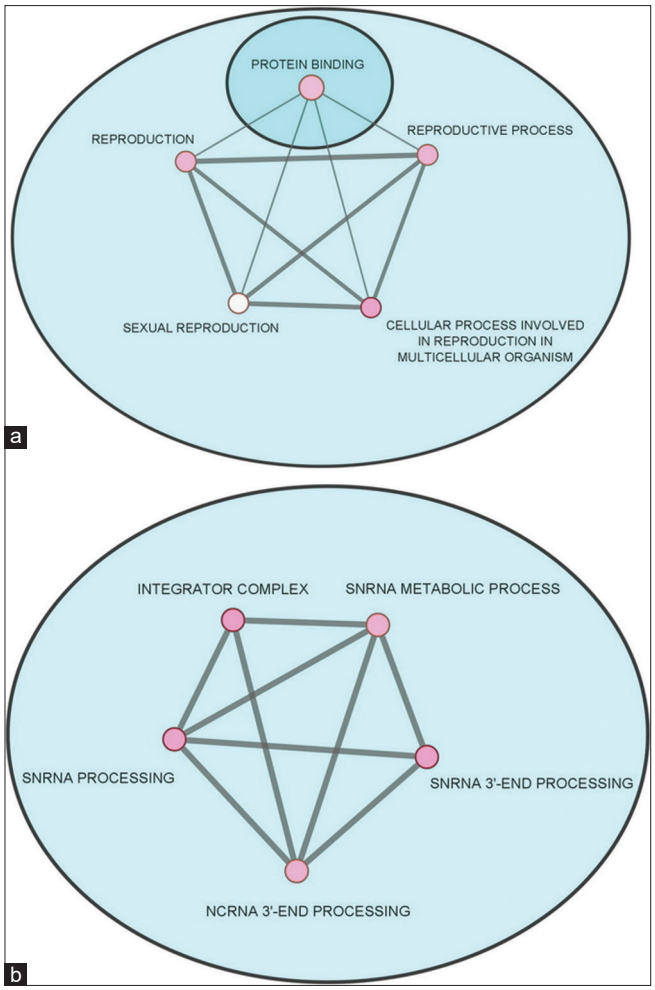

Figure 3: Enrichment pathway analysis. The enrichment map represents pathways enriched in differentially-expressed cancer/testis antigens in 443 samples of gastric adenocarcinoma. Nodes in the network show pathways and similar pathways with common genes are connected. Three clusters of nodes were identified: (a) reproduction of multicellular organism and protein binding, (b) end processing sn Ribonucleic acid (RNA)

proteins were involved in the integrator complex which is responsible for the biogenesis of small nuclear RNAs and enhancer RNAs.[23] KEGG pathway enrichment did not show any statistically significant pathway.

Co-expression network analysis

To predict the potential functional roles of normalized genes in STAD, after determining the optimal parameter ($\beta = 5$), the WGCNA algorithm was used to convert the correlation coefficient of a gene pair into the adjacent coefficient. The adjacency matrix was used to determine nodes, edges, and edges weight. The network was imported to Cytoscape. A CTA sub-network was detected after analyzing the gene-gene network [Figure 4].

Survival analysis

The Kaplan-Meier curves were plotted utilizing log-rank calculations. The medians of normalized gene counts were found. Samples were categorized into up-and down-regulated

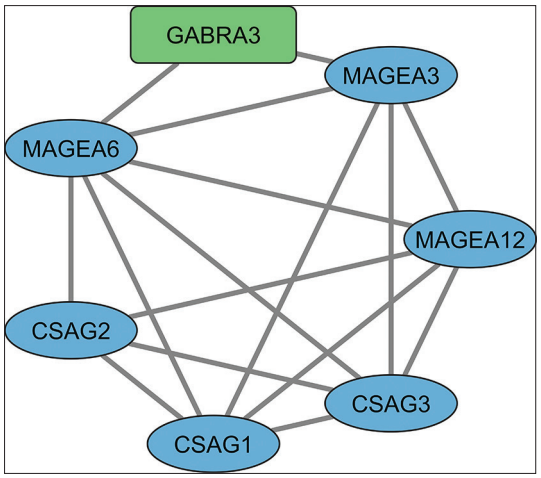

Figure 4: A sub-network of cancer/testis antigens genes extracted from weighted gene correlation network analysis. Blue ovals represent CTA genes and the green rectangle represents a non-CTA protein-coding gene. MAGEA3: Melanoma antigen gene (MAGE) family member A3, MAGEA6: MAGE family member A6, MAGEA12: MAGE family member A12, CSAG1: Chondrosarcoma associated gene 1, CSAG2: Chondrosarcoma associated gene 2, CSAG3: Chondrosarcoma associated gene 3 and GABRA3: Gamma-aminobutyric acid type A receptor subunit alpha3 are the members of this subnetwork

gene groups based on the median. The potential association of differentially-expressed CTA genes with the overall survival (OS) of STAD was then investigated. Up-regulation of MAGEA11, MAGEC3, Per ARNT SIM domain containing 1 (PASD1), Placenta-specific protein 1 (PLAC1), and sperm protein associated with the nucleus, X-linked B1 (SPANXB1) were significantly associated with the poorer OS of STAD patients [Figure 5].

Discussion

CTAs are auto-antigens typically expressed in germline tissues and many tumors.[10,24] The testis is an immune-privileged site with a blood-testis barrier.[25] This barrier restricts the immune system access to the testis. The exact functions of CTAs are not recognized. Nevertheless, they participate in transcription regulation, mitotic fidelity, and protein degradation.[10] In malignancies, these genes may assist in tumor cell fitness and cancer stem cells proliferation.[10]

Up-regulation of CTAs in TCGA-STAD tumoral samples impelled us to conduct a survey of CTAs in gastric cancer. We gathered a comprehensive list of 280 CTAs genes from four different databases. Almeida *et al*. provide an inclusive database of CTA genes.[15] However, noncoding and some newly identified genes are not included.

We identified differentially-expressed RNAs in the TCGA gastric cancer cohort. MAGEA family members are the most up-regulated DEGs and CTAs in the present study. Furthermore, WGCNA was conducted to recognize highly correlated RNAs. MAGEA3, 6, and 12, in addition to chondrosarcoma-associated gene1 (CSAG1), CSAG2, and CSAG3, were CTAs that

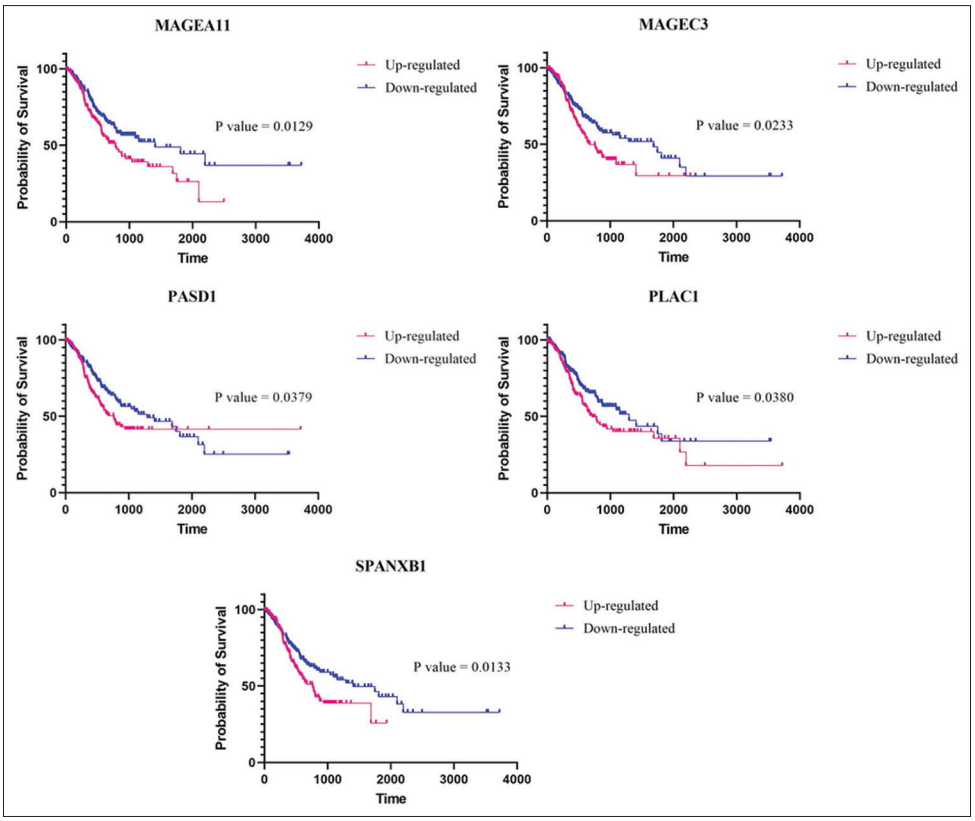

Figure 5: Overall survival related to up-regulated genes in gastric cancer. Kaplan–Meier survival curves estimate the overall survival of stomach adenocarcinoma patients with up-regulation of melanoma-associated antigen A11 (MAGEA11), melanoma-associated antigen C3 (MAGEC3), Per ARNT SIM D1 (PASD1), Placenta-specific protein 1 (PLAC1), and sperm protein associated with the nucleus, X-linked B1 (SPANXB1). The red line shows the up-regulated group, and the blue line indicates the down-regulated group. Statistical significance between the two groups was calculated using the log-rank test and determined when the $P < 0.05$

participated in a subnetwork in gastric cancer. Moreover, the GABRA3 is co-expressed with MAGEA3 and MAGEA6. In 2019, Fain *et al*. found that the GABRA3 gene is located near MAGEA3 and MAGEA6 locus.[26] Further investigation revealed that DNA demethylation agents activated MAGEA6 and GABRA3.[26] Hypomethylation of the MAGEA6 promoter activates MAGEA6 and GABRA3 in lung and melanoma tumors.[27] Endo *et al*. studied MAGEA6 in gastric cancer. They observed that MAGEA6 up-regulation indicates poor prognosis and recurrence.[28] Tsang *et al*. found that MAGEA6 up-regulation represses autophagy that promotes pancreatic cancer onset.[29] Oh *et al*. investigated the MAGEA12 in breast cancer and found that it promotes malignancy.[30] MAGEA12 expression is contributed to histone modifier proteins. This protein up-regulation attributes to epigenetic modifications.[30] The functional enrichment analysis of differentially-expressed CTA genes showed that MAGEA3, 6, 12, and CSAG3 are involved in binding proteins. Maxfield *et al*. reported that CSAG1 and CSAG3 might contribute to cell reproduction and tumor growth, despite not being involved in spermatogenesis.[31] Sapkota *et al*. identified that knockdown of CSAG1 disrupted the integrity of mitotic centrosome in cells with defective P53.[32]

CTAs are self-antigens that stimulate the immune system, i.e., T-cell mediated immune response.[24] Although CTAs have antigenicity properties, their roles in tumors are obscure. Studies reveal that MAGEA3 confers proliferation and chemoresistance.[30,33,34] Wang *et al*. investigated Sitagliptin in gastric cancer. They found that oral hypoglycemic agent suppresses expression of MAGEA3 and thus gastric cancer cell proliferation via AMPK/YAP/MAGEA3 pathway.[35] Our study showed an association between poorer OS in gastric cancer patients and MAGEC3 up-regulation. Wu *et al*. depicted that MAGEC3 activates epithelial-mesenchymal transition and consequently esophageal squamous cell carcinoma aggressiveness via immunosuppression.[36] PASD1 is an immunogenic protein in colorectal cancer that encourages cell proliferation in glioma cells.[37,38] In gastric cancer patients, this protein is up-regulated, and this up-regulation has a relationship to OS.

SPANXB1 is enriched in reproduction tissue pathways and involves protein binding. In triple-negative breast cancer, SPANXB1 interacts with SH3GL2 metastasis repressor. Overexpression of SPANXB1induces migration and invasion.[39] We also noticed that up-regulation of SPANXB1 contributes to OS in gastric cancer.

Our results showed that CTAs could be used as potential prognostic biomarkers in gastric cancer. WGCNA offered that the five members of cancer/testis antigen were co-expressed,

and these genes participate in cell proliferation, invasion, and metastasis. To our best knowledge, CSAG1, CSAG2, and CSAG3 roles in gastric cancer have not been studied. These genes can be pivotal biomarkers in gastric cancer. Survival analyses suggest that MAGEA11, MAGEC3, PASD1, PLAC1, and SPANXB1 have potential roles in gastric cancer development.

In conclusion, studies show that expression patterns of CTAs are restricted in normal tissues and different tumors. These genes are involved in stem cell development in normal and cancerous tissues. However, the underlying mechanisms are not fully understood. *In vitro* studies would be valuable for exploring the function of CTAs in tumors. Besides, investigating their expression patterns in cancer at different stages would provide clues to finding the roles of CTAs in cancer development and to find exclusive diagnostic/prognostic biomarkers.

Supplementary materials

All supplementary data are available in the Mendeley Data repository as: Mendeley Data, V1, doi: http://dx.doi.org/10.17632/h4gjg7jyyx.1.

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Conflicts of interest

There are no conflicts of interest.

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