Transcriptome sequencing identifies key pathways and genes involved in gastric adenocarcinoma

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Abstract. The present study aimed to investigate the key pathways and genes associated with gastric adenocarcinoma via transcriptome sequencing. Five pairs of gastric adenocarcinoma tissue and normal tumor-adjacent tissue were harvested. After sequencing, raw data were processed and differentially expressed genes (DEGs) between tumor and control groups were screened, followed by functional enrichment analysis and gene clustering analysis. The effect of DEGs on patient prognosis was analyzed on the basis of the survival data from gastric adenocarcinoma patients in The Cancer Genome Atlas database. Several genes were validated through reverse transcription-quantitative polymerase chain reaction. In total, 1,477 upregulated and 282 downregulated DEGs were screened in tumor groups. These genes were segregated into four clusters. Genes in cluster 1 were significantly involved in metabolism of xenobiotics by cytochrome P450, genes in cluster 2 were majorly involved in apoptosis, tight junction formation, and platelet activation, genes in cluster 3 were primarily enriched in the p53 signaling pathway and genes in cluster 4 were significantly enriched in the insulin resistance pathway. Furthermore, 15 DEGs significantly influenced prognosis, including F2R, CTHRC1, and RASGRP3. The expression levels of CYP2B6, MAPK13, CTHRC, RASGRP3 and PYGM were consistent with our analysis results. In conclusion, pathways for metabolism of xenobiotics via cytochrome P450, apoptosis, tight junction formation, platelet activation, and insulin resistance may serve important roles in the progression of gastric adenocarcinoma. Notably, CTHRC1 and RASGRP3 may serve as key prognostic markers.

Introduction

Gastric cancer is the third leading cause of cancer-related mortality worldwide (1). Adenocarcinoma constitutes the majority, approximately 90%, of gastric cancer cases (2). Adenocarcinoma is a malignant epithelial tumor that invades the gastric wall, infiltrates the muscularis mucosae, submucosa, and muscularis propria. Since early gastric cancer yields few symptoms, gastric cancer is usually advanced at diagnosis, which is difficult to cure (3). Advanced-stage gastric adenocarcinoma has a poor prognosis and the spontaneous median survival ranges from 3 to 6 months (4). Achieving a detailed understanding of the genetics and molecular pathogenesis of gastric adenocarcinoma may help improve patient outcomes.

Altered regulation of gene expression programs is important for tumors to express different cancer biomarkers (5,6). A Recent study has achieved considerable progress in identifying the key molecular mediators of gastric cancer. For instance, gene changes in Cadherin 1 expression, AT-rich interaction domain 1A, and ras homolog family member A, as well as some deregulated pathways including AMPK/HNF4a/Wnt5a pathways are associated with gastric malignancy and progression (7-10). Although several genes have been reported, a large proportion of gastric cancer patients have none of these genes in their cancer genome. Therefore, further detailed genomic characterization of gastric cancer patients is required.

Transcriptome sequencing is a rapidly developing approach to provide an unprecedented global view of the transcriptome, thereby revealing the entire transcriptional landscape (11,12). In this study, we used transcriptome sequencing to compare gene expression changes in five pairs of gastric adenocarcinoma tissue and normal tumor-adjacent tissue. Transcriptome sequencing data were then analyzed in silico. The present study aimed to further explore genetic and biochemical markers associated with gastric adenocarcinoma.

Materials and methods

Samples. Five pairs of gastric adenocarcinoma tissue and normal tumor-adjacent tissue were obtained from five gastric adenocarcinoma patients (Table I). The tissue samples were snap-frozen and stored in liquid nitrogen. All patients provided informed consent before the study. In addition, all procedures in this study were approved by our hospital’s protection of human ethics committee.

RNA isolation, library preparation, and sequencing. Total RNAs were isolated from tumor and paired normal
tumor-adjacent tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA integrity was detected using the RNA Nano 6000 Assay kit (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA concentration and purity were assessed using Qubit®RNA Assay kit in Qubit®2.0 Fluorometer (Thermo Fisher Scientific, Inc.), and NanoPhotometer® spectrophotometer (Implen, Inc., Westlake Village, CA), respectively. mRNA was then purified using oligo (dT) magnetic beads, and the high-quality mRNA were pooled to generate a cDNA library, using the NEBNext® Ultra™ RNA Library Prep kit for Illumina®. Briefly, mRNA was fragmented into small pieces, followed with first-strand cDNA synthesis with random hexamer-primers. Thereafter, double-stranded cDNA was synthesized and purified with AMPure XP beads. Purified double stranded cDNA was then subjected to end repair, dA tailing, and adaptor ligation. After size selection using AMPure XP beads, cDNA libraries were constructed and sequenced using the Illumina HiSeq 4000 platform.

The data are deposited at National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession no. SRP119102).

**Raw read quality control and reference genome alignment.** Raw reads were quality-filtered to obtain clear data via removal of adaptor sequences, ambiguous or low-quality reads and reads with more than 5% N, using Fastx toolkit version 0.0.13 and Prinseq-lite version 0.20.4 (13). The clear reads were aligned with the human reference genome GRCh38 using Tophat (version 2.0.8, http://htseq.readthedocs.io/) (14). The default parameters were the following: read-mismatches, 2; read-gap-length, 2; and min-anchor, 8. Thereafter, the clear reads were annotated using HTseq (version 0.6.1, http://www-huber.embl.de/HTSeq) (15) on the basis of the GRCh38 gene annotation information in gene code.

**Identification of differentially expressed genes (DEGs) analysis.** The mRNA read counts were transformed into log-counts per million (logCPM) using edger (version 3.4, http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) in R (16,17). Genes with low expression values were excluded. The obtained genes were normalized using trimmed mean of M-values (TMM) algorithm. Thereafter, the normalized data were transformed into a gene expression matrix, using the voom method (18) in limma (version 3.32.5, http://bioconductor.org/packages/release/bioc/html/limma.html) package (19). DEGs were determined using empirical Bayes linear model and the P-value for the expression of all genes was obtained. A P-value <0.05 and\( \log_{2}(\text{fold-change})\geq1 \) were set as the cut-off values. The heatmap of DEGs was clustered using pheatmap (version 1.0.8, https://cran.r-project.org/web/packages/pheatmap/package in R (20).

**Functional and pathway enrichment analyses.** We used clusterProfiler (version 3.4.4, https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) to implement Gene Ontology (GO) (21) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (22) analyses for up- and down-regulated DEGs. The Benjamini and Hochberg (BH) method-adjusted P-value <0.05 was used as cut-off criteria.

**Gene clustering analysis.** On the basis of the gene expression values in cancer and control groups, we applied clustering analysis for DEGs using ConsensusClusterPlus (version 1.40.0, https://www.bioconductor.org/packages/release/bioc/html/ConsensusClusterPlus.html) in R (23). The clustering method was K-means algorithm with the Euclidean distance. The number of clusters was identified through cumulative distribution function (24).

**PPI network and pathway analyses of clustering module.** Based on the clustering modules obtained, we utilized the Search Tool for the Retrieval of Interacting Genes (STRING, Version 10.0, http://www.string-db.org/) (25) database to analyze protein-protein interactions among DEGs. The Cytoscape (version 3.4.0, http://www.cytoscape.org/) (26) software was used to visualize the PPI network. The topological characteristics of nodes in the network were analyzed using CytoNCA (version 2.1.6, http://apps.cytoscape.org/apps/cytonca) (27). Based on the topological properties scores of nodes, hub proteins (28) were selected. Additionally, we performed KEGG pathway enrichment analysis for genes in clustering modules.

**Prognostic analysis.** The effect of DEGs on patient prognosis was analyzed using the stomach adenocarcinoma (STAD) survival data in The Cancer Genome Atlas (TCGA) database.

### Table I. Characteristics of patients.

| Samples | Sex | Age (yr) | Height (cm) | Weight (kg) | Stage |
|---------|-----|---------|------------|-------------|-------|
| G1      | Male | 64      | 170        | 55          | T1N0M0|
| G2      | Female | 78     | 149        | 60          | T2N2M1|
| G3      | Male | 63      | 172        | 65          | T4aN2M0|
| G4      | Female | 84     | 155        | 55          | T1N0M0|
| G5      | Male | 74      | 171        | 65          | T1N0M0|

| Primer name | Sequences (5’-3’) |
|-------------|-------------------|
| GAPDH-hF    | TGACACCTTGTATCGTGGAGG |
| GAPDH-hR    | AGGCAGGGATGATTTCTGAGAG |
| CYP2B6-hF   | TGGAGGCAGGGATGATTTCTGAGAG |
| CYP2B6-hR   | GCTGACACCTCAGGACGAGG |
| MAPK13-hF   | TGGAACATCCAGGAGCCCAA |
| MAPK13-hR   | CGTCCGCTGTCGTCGTCGTC |
| F2R-hF      | TGACCCGGGGATCTAAGGTG |
| F2R-hR      | CCGCCAGGATGAGGAGCATAC |
| CTHRC1-hF   | TTTCCCTACAAGATCTCCC |
| CTHRC1-hR   | TCAGTTTTCGTACCTCCCT |
| RASGRP3-hF  | G4 Female 84 155 55 T1N0M0 |
| RASGRP3-hR  | G4 Female 84 155 55 T1N0M0 |
| PYGM-hF     | G4 Female 84 155 55 T1N0M0 |
| PYGM-hR     | G4 Female 84 155 55 T1N0M0 |
In TCGA database, we downloaded the mRNA-Seq and clinical data. In total, 371 samples displayed both gene expression values and clinical data, which were selected for prognostic analysis. Briefly, each DEG was divided into two groups in accordance with its expression level (relative to the median of expression value) in patients, followed by analysis using Kaplan-Meier (KM) survival curves. Significant differences between high- and low-expression groups were analyzed using the log-rank test.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) verification of the expression of key genes. The expression levels of several genes were detected using RT-qPCR based on the five pairs of gastric adenocarcinoma tissue and normal tumor-adjacent tissue. Briefly, total RNAs were isolated using a TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration and quality were determined on a TECAN infinite M100 PRO Biotek microplate reader (Tecan, San Jose, CA, USA). Then 0.5 μg of the total RNA was used from cDNA synthesis using the PrimeScript RT Master Mix (RR036A; Takara Biotechnology Co., Ltd., Dalian, China). RT-qPCR was performed using the SYBR Green kit (4367659; Thermo Fisher Scientific, Inc.) in the Viia7 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used in this study are listed in Table II.

Statistical analysis. Data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Differences in gene expression levels among different groups were analyzed by one-way analysis of variance. The least square difference test was used for post hoc analyses. P<0.05 was considered significant.

Results

Reference genome alignment. The reads mapped to the human reference genome (GRCh38). The alignment rates of ten samples ranged from 76.58% to 82.83% (data not shown).

Analysis of DEGs. In total, 1477 upregulated and 282 downregulated DEGs were screened out in tumor groups compared with the control. Clustering analysis revealed that DEGs could clearly distinguish between tumor and control groups, as shown in the heatmap (Fig. 1).

Functional enrichment analysis. Results of functional enrichment analysis are shown in Fig. 2. Upregulated DEGs were significantly associated with the binding of cell adhesion molecules, and cysteine-type endopeptidase activity, as well as the p53 signaling pathway, and TNF signaling pathway. Downregulated DEGs were significantly associated with the GO term ‘ion channel binding’, and pathway of ‘Vascular smooth muscle contraction’.

Gene clustering analysis. Using the consensus cluster algorithm, when k=4, the consensus matrix plot (Fig. 3A) presented
a clear distribution of high consistency and low consistency in classification. Moreover, the classification achieved the maximum stability when \( k = 4 \) (Fig. 3B). Furthermore, \( k = 4 \) was the largest \( k \) with an appreciable increase in consensus (Fig. 3C). Therefore, \( k = 4 \) was considered the optimal clustering number. Based on \( k = 4 \), four clusters were obtained. The number of genes in clusters 1-4 was 410 (upregulated), 567 (479 upregulated and 88 downregulated), 591 (588 upregulated and 3 downregulated), and 191 (downregulated), respectively.

**PPI network and pathway analyses of clustering module.** The genes in the four clusters were subjected to PPI network analysis. The PPI network of cluster 1 comprised 192 nodes (such as non-SMC condensin I complex subunit H) and 511 edges (Fig. 4); cluster 2, 440 nodes (such as mitogen-activated protein kinase 13 (MAPK13)) and 1,745 edges (Fig. 5); cluster 3, 385 nodes and 1,260 edges (Fig. 6); cluster 4, 40 nodes [glycogen phosphorylase, muscle associated (PYGM)] and 47 edges (Fig. 7). The top five genes with high degrees (hub genes) in the four networks are enlisted in Table III.

KEGG pathway enrichment analysis revealed that genes in cluster 1 were significantly involved in Maturity-onset diabetes among younger individuals, protein digestion and absorption, and xenobiotic metabolism via cytochrome P450; cluster 2, majorly involved in apoptosis, tight junction formation, and platelet activation; cluster 3, primarily enriched in the p53 signaling pathway, cell cycle, and TNF signaling pathway; cluster 4, significantly enriched in insulin resistance and neuroactive ligand-receptor interactions (Fig. 8).
Prognostic analysis. A total of 15 DEGs were identified to significantly influence patient prognosis (Table IV), including cystatin SA (CST2), coagulation factor II thrombin receptor (F2R), Collagen triple helix repeat containing 1 (CTHRC1), and RAS guanylreleasing protein 3 (RASGRP3). Furthermore, 9 genes were identified in cluster 3.

RT-qPCR verification of the expression of key genes. Expression levels of CYP2B6, MAPK13, F2R, CTHRC1, RASGRP3, and PYGM were determined using RT-qPCR. As shown in Fig. 9, CYP2B6, MAPK13, and CTHRC1 were significantly upregulated in tumor samples compared with controls (P<0.05). RASGRP3 was also upregulated in tumor tissue but the difference was not significant. Additionally, F2R and PYGM were significantly downregulated in tumor samples compared with controls (P<0.05). Taken together, the expression levels of CYP2B6, MAPK13, CTHRC1, RASGRP3 and PYGM were consistent with our analysis results.

Discussion

In total, 1,477 upregulated and 282 downregulated DEGs were screened out in tumor groups compared with the control. These genes were segregated into 4 clusters. Genes in cluster 1 were significantly involved in metabolism of xenobiotics via cytochrome P450. Genes in cluster 2 were majorly involved in apoptosis, tight junction formation, and platelet activation. Genes in cluster 3 were primarily enriched in the p53 signaling pathway. Genes in cluster 4 were significantly enriched in the insulin resistance pathway. Furthermore, 15 DEGs were identified to significantly influence patient prognosis, including F2R, CTHRC1, and RASGRP3.

Cytochrome P450 enzymes are predominantly hepatic enzymes involved in drug and xenobiotics metabolism (29). However, reactive intermediates are formed during the conversion of the parent compound to the hydrophilic conjugated product that is cleared via excretion. These intermediates could
cause genotoxicity and affect the checkpoint-signaling and stress-signaling pathways to cause aberrant cell growth and alter the cell cycle, thereby leading to tumor initiation (30). Interestingly, some cytochrome P450 family genes are correlated with the progression of gastric adenocarcinoma (31). The present study shows that three differentially expressed cytochrome P450 family genes (CYP2B6, CYP2C9, and CYP3A4) of cluster 1 were significantly enriched in xenobiotic metabolism via cytochrome P450 (hsa00980), suggesting that these DEGs may be involved in the development of gastric adenocarcinoma through xenobiotic metabolism via the cytochrome P450 pathway.

DEGs in cluster 2 were significantly enriched in apoptosis (hsa04210) and tight junction formation (hsa04530). These two pathways are associated with gastric tumorigenesis (32,33). Additionally, platelet activation (hsa04611) was also a significant pathway among genes of cluster 2, which was enriched by MAPK13 (a hub gene) and F2R (prognosis associated gene). MAPK13 encodes the p38d isofrom, which plays a role in the tumor initiation (34). Platelets are multifaceted cells, and circulating platelets can influence various pathophysiologic events (35). Platelets exacerbate tumor progression and metastasis (36,37). In 1968, Gasic et al (38) reported that thrombocytopenic mice are protected against metastasis, supporting the relevance of platelets in cancer progression. Together, pathways of apoptosis, tight junction formation, and platelet activation, as well as MAPK13 and F2R may play important roles in gastric adenocarcinoma. Nevertheless, the F2R expression detected in RT-qPCR was inconsistent with the analysis results. Therefore, further study are needed to investigate the role of F2R in gastric adenocarcinoma. Prognostic analysis revealed that most of the
obtained prognosis-associated genes were present in cluster 3, including \textit{CTHRC1} and \textit{RASGRP3}. \textit{CTHRC1} was first identified during screening of differentially expressed sequences between balloon-injured and normal rat arteries (39). It is overexpressed in several types of malignant tumors, including gastric cancer (40). Tang \textit{et al} (41) reported that CTHRC1 plays key functional roles in cancer progression by increasing cancer cell invasion and metastasis. \textit{RASGRP3} is a member of the RASGRP family that was initially reported to be present in the screen of genes whose overexpression induce fibroblast transformation (42). The involvement of the RasGRP family in cancer progression and development is proving to be extensive (43, 44). \textit{RASGRP3} could mediate the activation of the Ras signaling pathway, which plays a key role in carcinogenesis (45). Considering the critical roles of \textit{CTHRC1} and \textit{RASGRP3} in carcinogenesis and the present results, we considered the two genes as important prognostic markers in gastric adenocarcinoma.

\textit{PYGM}, a hub gene in the PPI network of cluster 4, was involved in the insulin resistance pathway (hsa04931). Insulin resistance is a pathological condition characterized by a decline in the efficiency of insulin signaling for the regulation of blood sugar (46). Insulin is a potent mitogenic agent, which can inhibit apoptosis and promote cell proliferation (47).

Figure 6. The protein-protein interaction network generated on the basis of genes in the cluster 3. The gray round node represents upregulated genes and white diamonds indicate downregulated genes.

Figure 7. The protein-protein interaction network generated on the basis of genes in the cluster 4. The white diamond node represents downregulated genes.
Table III. Top five differentially expressed genes with high degrees (hub genes) in four networks.

| Cluster  | Node number | Up DEGs | Down DEGs | Edge number | Degree top 5 gene |
|----------|-------------|---------|-----------|-------------|------------------|
| Cluster 1 | 192         | 192     | 0         | 511         | NCAPH, CDX1, DLGAP5, NCAPG, MNX1 |
| Cluster 2 | 440         | 375     | 65        | 1,745       | GGT1, MAPK13, ENO2, TRAP1, DUSP4 |
| Cluster 3 | 385         | 384     | 1         | 1,260       | AURKA, CDH17, MYBL2, CDC6, CFTR |
| Cluster 4 | 40          | 0       | 47        | 47          | GHR, PYGM, CSF3, STX1B, FAM149A |

Table IV. Differentially expressed genes that significantly affect patient prognosis.

| Names      | p     | High.median | Low.median | Regulated | Cluster |
|------------|-------|-------------|------------|-----------|---------|
| CST2       | 0.042178 | 22.17       | 46.22      | Up        | 1       |
| CTSV       | 0.03481  | 25.59       | 57.39      | Up        | 1       |
| MATN3      | 0.000184 | 21.98       | 68.99      | Up        | 1       |
| SYT12      | 0.040832 | 25.59       | 46.22      | Up        | 1       |
| F2R        | 0.013663 | 25.59       | 55.39      | Up        | 2       |
| AADAC      | 0.047637 | 25.69       | 55.39      | Up        | 3       |
| AGT        | 0.025573 | 26.02       | 55.39      | Up        | 3       |
| TMEM243    | 0.031481 | 22.17       | 55.39      | Up        | 3       |
| CTHRC1     | 0.001409 | 23.39       | 59.49      | Up        | 3       |
| F5         | 0.001542 | 21.42       | 55.39      | Up        | 3       |
| KYNU       | 0.011683 | 25.03       | 59.49      | Up        | 3       |
| MSC        | 0.028155 | 25.16       | 46.22      | Up        | 3       |
| RASGRP3    | 0.035342 | 25.59       | 42.51      | Up        | 3       |
| SLC7A7     | 0.025332 | 22.17       | 42.51      | Up        | 3       |
| ADPRHL1    | 0.043589 | 42.51       | 26.08      | Down      | 4       |

p represents statistical significance analyzed via a log-rank test; High.median, represents median survival time in the high expression group; Low.median, represents median survival time in the low expression group; Regulated, represents variations in the expression level trends of genes (Tumor vs. Control).

Figure 8. Kyoto Encyclopedia of Genes and Genomes pathways enriched by genes in the four clusters.
Trevisan et al (48) reported that the variables related to an increase in insulin resistance are related to an increased risk of death from colorectal cancer. Furthermore, Mu et al (49) reported that insulin resistance was a risk factor for endometrial cancer. Therefore, we speculated that PYGM may be implicated in gastric adenocarcinoma via the insulin resistance pathway.

In conclusion, the present study suggested that pathways of xenobiotic metabolism via cytochrome P450, apoptosis, tight junction formation, platelet activation, and insulin resistance as well as the enriched genes including CYP2B6, MAPK13, and PYGM may play important roles in the progression of gastric adenocarcinoma. Furthermore, CTHRC1 and RASGRP3 may serve as key prognostic markers for gastric adenocarcinoma patients.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
WHZ and ZBY contributed to the data collection and conducting the study, data analysis, and writing of the manuscript. HXZ and ZBY contributed to the study design, conducting the study, data analysis, and writing of the manuscript. GYZ contributed to data interpretation and discussion. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All procedures in this study were in accordance with the Declaration of Helsinki and approved by the protection of human ethics committee of Qilu Hospital of Shandong University.

Patient consent for publication
All patients provided informed consent for the study.

Competing interests
The authors declare that they have no competing interests.

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Figure 9. Relative expression levels of CTHRC1, CYP2B6, F2R, MAPK13, PYGM and RASGRP3 in gastric adenocarcinoma tissue and normal tumor-adjacent tissue determined by reverse transcription-quantitative polymerase chain reaction. Their expression levels are normalized against GAPDH. *P<0.05, **P<0.01.
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