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Original Article

Transcriptome profiling of cancers tissue in Chinese gastric patients by high-through sequencing

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Abstract: Gastric cancers (GC) have the high morbidity and mortality rates worldwide and there is a need to identify sensitive biomarkers for GC. Genome-wide screening of transcriptome dysregulation among cancer and normal tissues would provide insights into the molecular mechanism of GC initiation and progression. Up to date, high throughput sequencing technique has begun to innovate biomedical studies. RNA-seq method has become an advanced approach in medicine studies, which is capable of accurate detection of gene expression levels. In this work, we used RNA-seq data from tumor and matched normal samples to evaluate their transcriptional changes and further verified differentially expressed genes in larger samples. We totally identified 28 mRNAs up-regulation and 22 down-regulations between cancer and normal samples. Then, we selected five differentially expression gene to verify in large samples and chose CDH1 to detect protein expression levels. The results revealed CDH1, COX-2 and MMP were significantly higher expression, whereas the expression level of DPT and TGFBR2 were decreased in gastric cancer samples. Particularly, CDH1 was 36-fold higher expression in cancer sample. The result of WB also demonstrated CDH1 was highly expressed in validation cohorts. Furthermore, these genes are highly enriched in some gene ontology (GO) categories, such as "digestive system process", "secretion", and "digestion". This study provided the preliminary survey of the transcriptome of Chinese gastric cancer patients, which may be benefit for detection of altered gene and understanding basis in tumorgenesis.

Keywords: Gastric cancers, RNA-seq, differentially expressed gene, gene ontology

Introduction

Gastric cancer (GC), also known as stomach cancer, Early symptoms may include heartburn, upper abdominal pain, nausea and loss of appetite. Later signs and symptoms may include weight loss, yellow skin and whites of the eyes, vomiting, difficulty swallowing, and blood in the stoolamong others. The cancer may spread from the stomach to other parts of the body, particularly the liver, lungs, bones, lining of the abdomen and lymph nodes [1]. Diagnosis is usually by biopsy done during endoscopy. This is then followed by medical imaging to determine if the disease has spread to other parts of the body. The most common cause is infection by the bacterium Helicobacter pylori, which accounts for more than 60% of cases [2]. Certain types of H. pylori have greater risks than others. Other common causes include eating pickled vegetables and smoking. Globally stomach cancer is the fifth leading cause of cancer and the third leading cause of death from cancer making up 7% of cases and 9% of deaths. Up to date, therapeutics options for patients with gastric cancer are still limited, with some combination of surgery, chemotherapy, radiation therapy, and targeted therapy. So, understanding the molecular mechanisms underlying the carcinogenesis of GC is indispensable to cancer prevention, treatment and prognosis [3].

A challenge for the application of large scale functional genomics to cancer research is to identify the expression profile as a potential source of specific cancer genes useful as biomarkers. Some previous studies have investigated DEGs between tumor and normal tissues using high-throughput screening technologies, which to some extent provide numerous diagnostic and prognostic biomarkers. However, GC

are systems biology diseases and the heterogeneity and complexity of carcinogenesis complicate the marker identification process [4, 5]. Here in we comparative transcriptomic studies not only allow us to fill in the gap between driver mutations and pathological characteristics of tumor cells, but also facilitate identification of specific DEGs as potential biomarker for GC, and further comprehension the molecular basis of gene regulation.

With the development of next-generation sequencing, RNA-seq technology provides a powerful method to detect the transcriptome profile with high precision and inexpensive. The goal of our present study was to determine transcriptomes profile in GC tissues and compare it to the normal healthy gastric mucosa using RNA-seq. Comparative analyses of gene expression were performed to identify differentially expressed genes (DEGs) between gastric cancer and normal tissues. We have generated a lot of information on DEGs in Chinese gastric cancer versus normal tissues, which might provide beneficial information for the research for the molecular mechanism of carcinogenesis, detection of disease markers and the new targeted anti-cancer drugs.

Materials and methods

Subject samples

Tissue specimens used in this work, including tumor and distal normal tissues were prospectively collected between 2012 and 2015 in Departments of Gastroenterology and Surgery, Pudong Hospital-Affiliated to Fudan University Pudong Medical Center (Pudong, Shanghai). This study included a total of 33 control subjects and 33 GC patients, which were further divided into the transcriptome profiling groups-based on deep sequencing (GC, n=3 controls, n=3) and validation cohorts (GC, n=30; controls, n=30). All subjects were Han Chinese. Gastric biopsy samples were obtained from antral part of the stomach from control subjects who were referred for upper GI endos copy due to dyspeptic symptoms and had no previous history of malignancy and without autoimmune or inflammatory disease. GC tissue samples were obtained from surgical specimens immediately after removal from GC patients undergoing primary surgery with no preoperative irradiation and chemotherapy. Gastric adenocarcinoma in GC patients was confirmed by histology and classified according to Lauren into diffuse and intestinal types. Demographic and clinical characteristics were collected from cases and controls using a unified questionnaire.

Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each participant prior to the study. The Medical Ethics Committee of the Jinshan Hospital of Fudan University approved this study.

Tissue sample preparation and RNA extraction

Gastric tissue samples were stored in RNAlater (Solarbio, Beijing, China) at +4°C overnight and then later stored at -80°C. 30 mg of tissue was homogenized in sterile condition. Then, the total RNA was extracted from cancer and normal tissues with the TRIzol (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality (including the rate of 28 s/18 s and RNA Integrity Number) of the each RNA sample was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation and sequencing

10 μg of RNA for each sample was used to construct the Illumina sequencing library by the NEBNext mRNA Sample Prep Kit 1 (New England Biolabs, Ipswich, MA, USA). Briefly, total RNA was first selected using oligo-d(T) probes for poly-A messenger RNAs (mRNAs) and followed by thermal mRNA fragmentation. The fragmented RNA was subjected to complementary DNA (cDNA) synthesis and further converted into double-stranded cDNA. Upon end repairing, the cDNA product was ligated to Illumina Truseq adaptors and size selected using the 2% agarose gel to generate the average 300 bp cDNA libraries. The QIAquickPCR was then performed to measure the relative concentration of the library in order to determine the volume to use for sequencing. The RNA-seq library was sequenced on the Illumina HiseqTM 2500 (Illumina, Inc., San Diego, CA, USA) platform as paired-end reads to 100 bp using 1 lane (with a control lane on the same flow cell) at Novogene Bioinformatics Technology Co. Ltd (Beijing, China). The DGE libraries were generated by Illumina HiseqTM 2500 with single-end technology in a single

run. The average read length of 90 bp was generated as raw data.

Analysis of Illumina transcriptome sequencing results

The Illumina analysis pipeline (CASAVA 1.7) was used to process the raw sequencing data. All raw reads were filtered to remove the adaptor sequence, poly-N reads, low quality reads (50% of the bases had a quality value ≤5), empty reads (no tags between the adaptors), and reads with only 1 copy number (probably sequencing error) and low complexity. At the same time, the Q20 (percentage of bases with a Phred value of at least 20) and GC content of the clean data were summarized. At last, a total of 10 G bp of cleaned reads were produced. The cleaned sequencing reads were then aligned to the UCSC human reference genome using TopHat v1.0.12 which incorporates Bowtie v0.11.3 software to perform the alignment. In order to access the transcription abundance for each gene, the Cufflinks v1.0.3 was used to process the aligned reads from different samples. The gene transfer format (GTF) file for reference genome annotation that used in this analysis was retrieved from UCSC database. The expression level for each transcript was normalized to the reads per kilobase of exon model per million mapped reads (FPKM). The Cuffdiff was used to process the original alignment file produced by Tophat and GTF file for genome annotation to determine the differentially expressed genes. After applying Benjamini-Hochberg correction for multiple test, the false discovery rate (F.D.R) <0.05 was selected as the criteria for significant differences. We performed gene ontology (GO) and Pathway enrichment analysis to investigate the biological significance of those differentially expressed genes. This analysis was performed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID), which is a set of web-based functional annotation tool. The differentially expressed genes and all the expressed genes were submitted as the gene list and background list, respectively. The 1% cut-off of the false discovery rate (F.D.R) was used.

qRT-PCR expression validation

To evaluate the quality of the sequence assembly and expression profile, 5 differentially expressed gene were selected to amplify utilizing reverse transcription PCR (RT-PCR) and were quantified by real time quantitative PCR (qRT-PCR). For RT-PCR, 1 μg of total RNA from the transcriptome sample was reverse-transcribed in a 20 μL reaction system according to the manufacturer's protocol (PrimeScript TM RT Reagent Kit, TaKaRa, Japan). The PCR primers were designed based on the sequences from the gene by Primer Premier 5.0 software. Each reaction was carried out in a total volume of 20 μL with 1 μL cDNA, 10 μL SYBR Green I Master (LightCycler® 480 SYBR Green I Master, Roche Diagnostics Ltd, Lewes, UK), 0.5 μL/primer, and 9 μL ddH_2O. qRT-PCR was performed using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics Ltd, Lewes, UK). The qRT-PCR program was set The PCR program consisted of 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C, and a final 3 min at 72°C with Premix TaqTM Version2.0 (TaKaRa, Japan). Each sample was run in triplicate. The data were analyzed with automatic settings for assigning the baseline, and average Ct and SD values were calculated. The expression level of mRNA in the tissue was normalized to *β-Actin*. The results were calculated using the ΔΔCT method. The data were analyzed with automatic settings for assigning the baseline, and average Ct and SD values were calculated.

Western blot

Gastric tissue from control and case were homogenized in 300 ml of lysis buffer (50 mM Hepes; pH 7.5; 150 mM NaCl; 10 mM EDTA; 10 mM glycerophosphate; 100 mM sodium fluoride; 1% Triton X-100; 1 mM PMSF and PI cocktail). After centrifugation of the homogenate (20 000 g, 15 min), the supernatants were used for western blotting. Fifty micrograms of protein extracts from samples were suspended in Laemmli buffer (100 mM Hepes; pH 6.8; 10% b-mercaptoethanol; 20% SDS), boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel. After separation, proteins were electrically transferred onto a nitrocellulose membrane. The membrane was incubated with blocking solution (1×TBS; 0.05% TWEEN-20; 5% non-fat milk) at room temperature for 1 h and incubated overnight with primary antibodies raised against CDH1. After incubation with the corresponding secondary antibodies coupled to peroxidase (Santa Cruz Biote-

Table 1. Statistics of each sample transcriptome data

Sample Name	Total reads	Clean reads	Genome map rate	Gene map rate
Control 1	59,318,462 (100%)	51,352,541 (86.6%)	79.24%	73.17%
Control 2	60,891,232 (100%)	52.065,542 (85.48%)	78.41%	72.41%
Control 3	58,010,790 (100%)	51.167,261 (88.21%)	77.53%	71.62%
Case 1	57,390,348 (100%)	49.765,172 (85.91%)	76.33%	73.61%
Case 2	58,540,531 (100%)	51.152,986 (87.25%)	78.12%	74.51%
Case 3	59,643,118 (100%)	50.351,271 (85.12%)	77.29%	72.65%

Table 2. Correlations value between each two samples

Sample	Control 1	Control 2	Control 3	Case 1	Case 2	Case 3
Control 1	1	0.997	0.982	0.991	0.984	0.989
Control 2	0.994	1.000	0.978	0.000	0.985	0.993
Control 3	0.995	0.995	1.000	0.985	0.982	0.994
Case 1	0.986	0.995	0.981	1.000	0.987	0.987
Case 2	0.994	0.997	0.972	0.971	1.000	0.973
Case 3	0.997	0.993	0.993	0.975	0.989	1.000

chnology), proteins were visualized using an enhanced chemiluminescence ECL Plus immunoblotting detection system (Amersham Biosciences Europe GmbH, Freiburg, Germany). The intensity of the immunoreactive bands was quantified using a blot analysis system (Bio-Rad Laboratories, Marne la coquette, France) and β-Actin was used as a loading control. Commercial markers (Seeblue pre-stained standard, Invitrogen) were used as molecular weight standards.

Statistical analysis

The FPKM data was analyzed using t-test and Benjamini Hochberg correction for false discovery rate such that differential expression was considered to be significant with a $P<0.01$. The data was normalized using rank invariant normalization and analyzed using the HTqPCR package. The validation and plasma qPCR expression data was analyzed using nonparametric Mann-Whitney U-test. A Benjamini Hochberg adjusted $P<0.05$ was considered to be statistically significant.

Results

Transcriptome sequencing

The cancer and normal samples were subjected to massively parallel paired-end cDNA sequencing. We totally obtained 178.2 and 175.4 million raw reads of 100 bp length in the normal and cancer tissues from Illumina sequencing respectively. Then, all of which were filtered by removed low quality reads and reads containing N and adaptor sequences. The remaining reads are called "clean reads" and used for downstream bioinformatics analysis. We use BWA to map clean reads to genome reference (the UCSC human reference genome hg.19) and use Bowtie to gene reference. The unique match reads for subjected samples were 154.5 and 151.4 million raw reads. The average coverage of sequencing depth was nearly 600 times of human transcriptome (30 Mbp and approximately 1% of the hg.19, based on the total length of the uniquely annotated exon region in the Ensembl database). The detail result is shown in **Table 1**.

Differentially expressed genes

Next, we detected the gene expression level and identified the differentially expressed genes between case and control samples using a software package: RSEM. RSEM computes maximum likelihood abundance estimates using the Expectation-Maximization (EM) algorithm for its statistical model, including the modeling of paired-end (PE) and variable-length reads, fragment length distributions, and quality scores, to determine which transcripts are isoforms of the same gene. FPKM method was used in calculated expression level. We totally measured 14,318 and 14,694 expressed genes by postulating that the FPKM value was greater than one among any samples of each group. Our analysis contained the majority of the annotated human genes. Then, we investigated the correlation of the gene expression between each two samples. The results showed that the correlation of gene expression level among samples were highly correlated (Pearson correlation coefficient r=0.92), suggesting the experiments were reliable and the samples chosen were reasonable (**Table 2**).

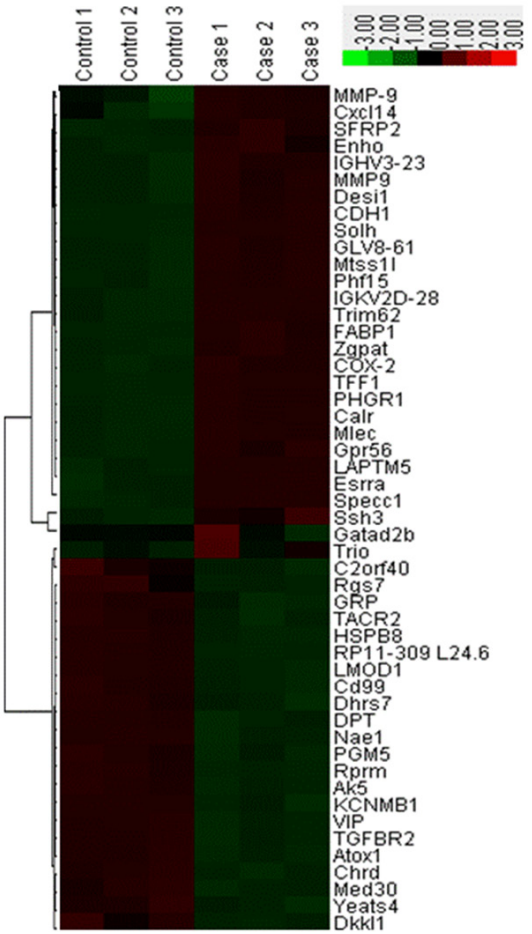

Figure 1. Heat map and cluster analysis of the 50 differentially expressed mRNAs in control and gastric cancer samples. Red represents high relative expression levels: Green represent low relative expression levels.

In order to refine our analysis, we strengthened our selection criteria with the threshold of F.D.R≤0.05 and Fold change ≥3. These stringent criteria generated a list of 28 mRNAs up-regulation and 22 down-regulation between cancer and normal samples. Which were clustered in **Figure 1**. The gene ranked the top five difference expression between normal and cancer samples is cadherin-1 gene (CDH1), cyclooxygenase COX-2), matrix metalloproteinase-9 (MMP-9), Dermatopontin (DPT) and TGFBR2. We further confirmed high-through sequencing result in validation cohorts, included samples participation in deep sequencing. Five difference expression gene between normal and cancer samples were chosen to verify by qRT-PCR. The results revealed CDH1, COX-2 and MMP were significantly higher expression, whereas the expression level of DPT and TGFBR2 were decreased in gastric cancer samples (**Figure 2**). Particularly, CDH1 was 36-fold higher expression in cancer sample. Which has been previously reported to have significantly altered expression in gastric cancer samples. The most significant downregulated genes in cancer sample are DPT gene, encoding dermatopontin protein. Moreover, result of WB also demonstrated CDH1 was highly expressed in validation cohorts (**Figure 3**).

Functional enrichment analysis of DEGs

To better understand the biological function of these DEGs, a gene ontology (GO) enrichment analysis was performed. Gene Ontology (GO), which is an international standard gene functional classification system, offers a dynamic-updated controlled vocabulary, as well as a strictly defined concept to comprehensively describe properties of genes and their products in any organism. GO has three ontologies: molecular function, cellular component and biological process. The basic unit of GO is GO-term. Every GO-term belongs to a type of ontology. GO enrichment analysis provides all GO terms that significantly enriched in a list of DEGs, comparing to a genome background, and filter the DEGs that correspond to specific biological functions. This method firstly maps all DEGs to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for every term, then uses hypergeometric test to find significantly enriched GO terms in the input list of DEGs, based on 'GO: Term Finder'. In our work, only biological process and molecular function categories were considered. The functional enrichment work was performed using an online tool, DAVID. With the threshold of F.D.R<0.05, we found that all differentially expressed genes were categorized into 12 functional categories (**Table 3**). For example, these over-represented GO categories include "digestive system process", "regulation of body fluid levels", "secretion", and "digestion".

Discussions

Our work provided a comprehensive insight into the transcriptome of gastric cancer and normal tissues and further verified DEGs expression among large samples. Using a whole transcriptome sequencing technology (RNA-seq), we were able to evaluate the levels of DEGs in

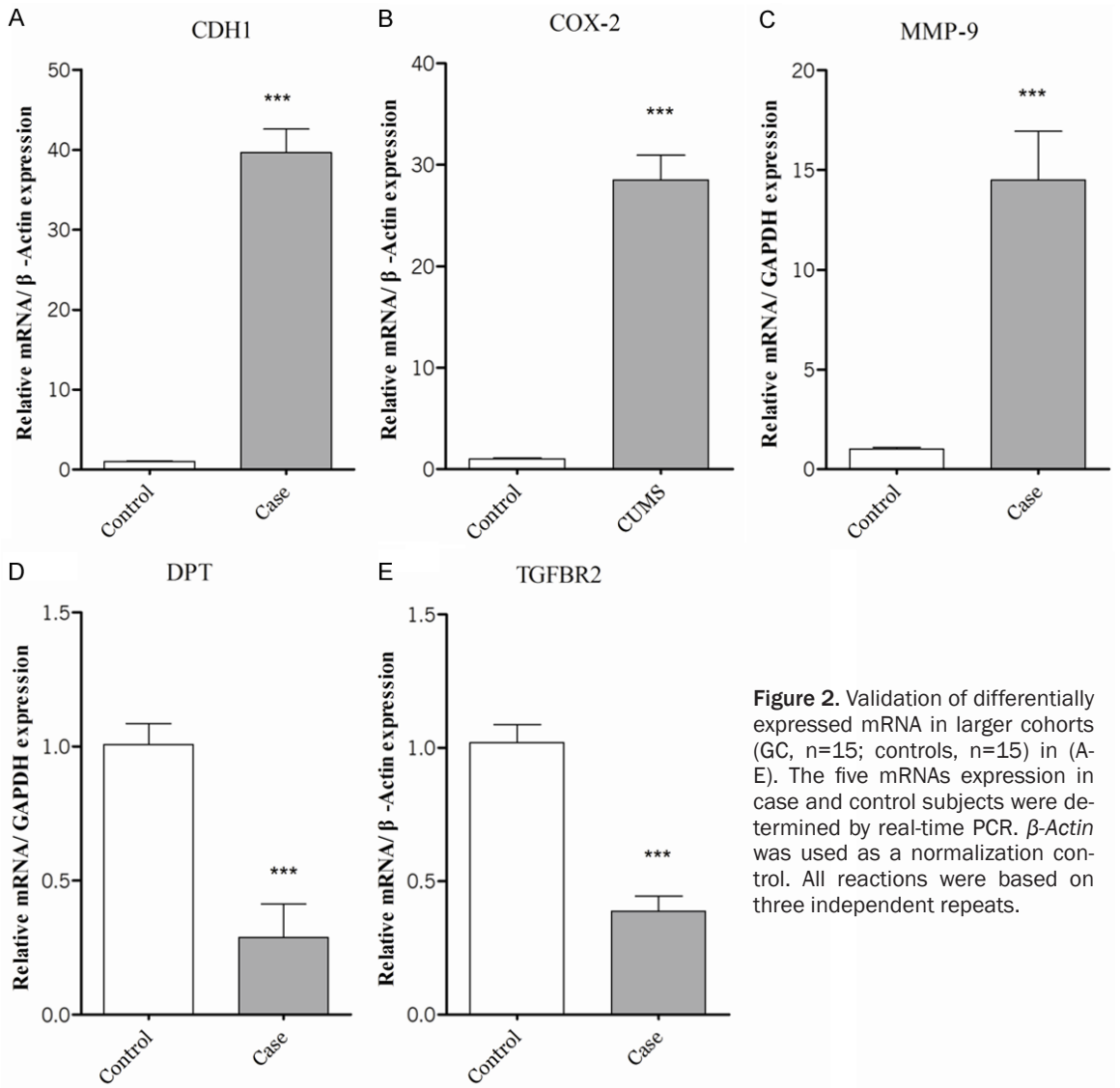

Figure 2. Validation of differentially expressed mRNA in larger cohorts (GC, n=15; controls, n=15) in (A-E). The five mRNAs expression in case and control subjects were determined by real-time PCR. *β-Actin* was used as a normalization control. All reactions were based on three independent repeats.

association with gastric cancer. Our work also offered new insights into the molecular mechanisms underlying GC, thereby beneficial for the diagnosis and treatment of GC.

For the whole transcriptome sequencing, we used an Illumina Hiseq 2500 platform with 90-bp sequencing reads length. We totally obtained more than 350 million raw reads, which has been previously reported to deliver sufficient sequencing coverage for transcriptome profiling. The rate of 98% of sequencing reads that map to the hg19 met quality standards of the RNA-seq technique. Hence, our mRNA-seq data provided a good representation of expressed genes in the human genome. Moreover, we further verified RNA-seq data in large samples by qRT-PCR and western blot. The result of qPCR and WB also were consistent with high-throughput sequencing results, which indicate high-throughput-sequencing may be becoming a novel approach to precision medical treatment of cancer.

We totally verified many DEGs and isoforms in gene expression in the gastric cancer. Many of these genes identified are known to be involved in numerous cancers. Based on our results, 12 GO categories have been confirm to over-represent among these DEGs such as “digestive system process”, “regulation of body fluid levels”, and “secretion (**Table 3**). Consistent with previous results, whole transcriptome analysis identified DEGs with biological functions that were

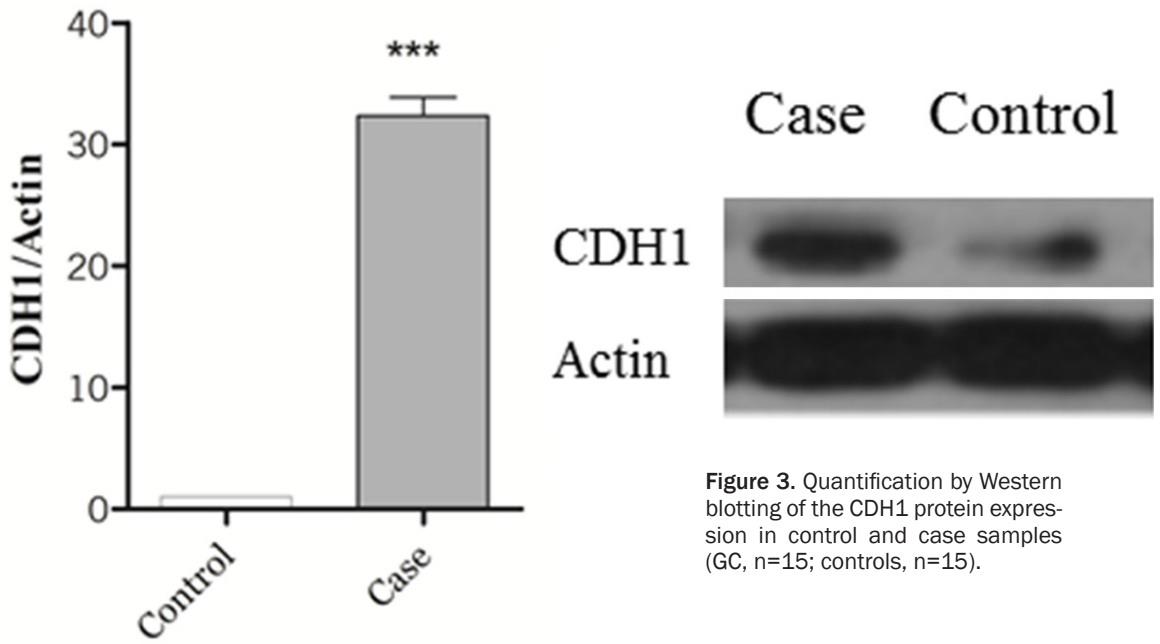

Figure 3. Quantification by Western blotting of the CDH1 protein expression in control and case samples (GC, n=15; controls, n=15).

Table 3. Enriched GO categories of DEGs

Category	GO ID	Go term	Cluster frequency	Genome frequency of use	Corrected *P*-value
BP	GO:0022600	Digestive system process	4 out of 50 genes, 8%	19 out of 15332 genes, 0.1%	0.001239
	GO:0050878	Regulation of body fluid	6 out of 50 genes, 12%	835 out of 15332 genes, 5.4%	0.001807
	GO:0046903	Secretion	8 out of 50 genes, 16%	143 out of 15332 genes, 0.9%	0.002405
	GO:0007586	Digestion	5 out of 50 genes, 10%	253 out of 15332 genes, 1.7%	0.002728
	GO:0007155	Cell adhesion	13 out of 50 genes, 26%	373 out of 15332 genes, 2.4%	0.003968
	GO:0022610	Biological adhesion	14 out of 50 genes, 28%	2954 out of 15332 genes, 19.3%	0.0041102
	GO:0007267	Cell-cell signaling	14 out of 50 genes, 28%	3787 out of 15332 genes, 24.7%	0.003139
	GO:0007967	System process	13 out of 50 genes, 26%	3787 out of 15332 genes, 24.7%	0.00294
MF	GO:0005184	Neuropeptide hormone	6 out of 50 genes, 12%	24 out of 15332 genes, 0.2%	0.0147
	GO:0005509	Calcium ion binding	20 out of 50 genes, 40%	4489 out of 15451 genes, 29.1%	0.0152256
	GO:0005179	Cytokine activity	10 out of 50 genes, 20%	3537 out of 15451 genes, 22.9%	0.070316
	GO:0003823	Symporter activity	12 out of 50 genes, 24%	2111 out of 15451 genes, 27%	0.0431

associated with cell adhesion. CDH1 gene shows the most significant changes between normal and cancer sample, with a 36-fold higher expression among cancer sample. Similarly, the level of CDH1 protein was also significantly higher expression in GC samples (**Figure 3**). CDH1 gene is a classical member of the cadherin superfamily [6-8]. Sequence mutations occurred in this gene are always correlated with gastric cancer. Moreover, loss of function is thought to contribute to progression in gastric cancer by increasing proliferation, invasion, and/or metastasis [9-13]. The expression dysregulation of CDH1 gene is tightly associated with cancer progression and metastasis and decreased the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility [14-18].

COX-2 with 8,3 kb length located on chromosome 1, 1q25.2~25.3, is consists of 10 exons and 9 introns, encoding 603 or 604 amino acids. However, it is almost not expressed in normal physiological conditions [19-23]. Several studies have shown that COX-2 gene was one of the early growth response gene and can be widely vessels inside and outside activator (such as interleukin 15-serotonin transforming growth factor etc.), resulting in tumorigenesis [24-28]. Sun reported in superficial

gastritis (100%), atrophic gastritis (35.7%) of intestinal metaplasia (37.8%) of the stomach dysplasia (41.7%) and gastric cancer (69.5%) of COX-2 positive the expression was gradually increasing trend. COX-2 is thought to be an early event in gastric carcinogenesis [29].

Matrix metallopeptidase 9 (MMP-9), also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B (GELB), is a matrixin, a class of enzymes that belong to the zinc-metalloproteinases family involved in the degradation of the extracellular matrix. In humans the MMP9 gene encodes for a signal peptide, a propeptide, a catalytic domain with inserted three repeats of fibronectin type II domain followed by a C-terminal hemopexin-like domain [30]. One of MMP-9's most widely-associated pathologies is the relationship to cancer, due to its role in extracellular matrix remodeling and angiogenesis. For example, its increased expression was seen in a metastatic mammary cancer cell line. Gelatinase B plays a central role in tumor progression, from angiogenesis, to stromal remodeling, and ultimately metastasis [31]. However, because of its physiologic function, it may be difficult to leverage Gelatinase B inhibition into cancer therapy modalities. However, Gelatinase B has been investigated in tumor metastasis diagnosis-Complexes of Gelatinase B/Tissue Inhibitors of Metalloproteinases are seen to be increased in gastrointestinal cancer and gynecologic malignancies. MMPs such as MMP9 can be involved in the development of several human malignancies, as degradation of collagen IV in basement membrane and extracellular matrix facilitates tumor progression, including invasion, metastasis, growth and angiogenesis [32]. Several studies detected MMP9 expression in gastric cancer tissues was significantly higher than that in normal gastric mucosa and serosa invasion, and MMP9 persons were significantly higher than those without serosal invasion by flow cytometry [33-35]. Differentiated group of lymph node metastasis was significantly higher than those without lymph node metastasis ($P \leq 0.05$). Additionally, Shan et al. successfully suppressed the expression of MMP9 in poorly differentiated gastric cancer cell line BGC-823 by pGenesil carrier, which subsequent laid the foundation for in vivo experiments and gene therapy [36, 37].

Dermatopontin is a protein that in humans is encoded by the DPT gene. Dermatopontin is an extracellular matrix protein with possible functions in cell-matrix interactions and matrix assembly. The protein is found in various tissues and many of its tyrosine residues are sulphated. Dermatopontin is postulated to modify the behavior of TGF beta through interaction with decorin [38]. Transforming growth factor, beta receptor II gene encodes a member of the serine/threonine protein kinase family and the TGFB receptor subfamily. The encoded protein is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with another receptor protein, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation. Mutations in this gene have been associated with Marfan syndrome, Loeys-Deitz aortic aneurysm syndrome, Osler-Weber-Rendu syndrome, and the development of various types of tumors [39, 40]. Alternatively spliced transcript variants encoding different isoforms have been characterized [41, 42].

In summary, based on the RNA-seq technology, sequencing reads were generated to profile the gastric cancer transcriptome. It provided wealth information on DEGs in case-control samples, which might be benefit for other studies and lead to vastly improved methods for detection and therapy gastric cancer.

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Disclosure of conflict of interest

None.

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