Comprehensive bioinformatics analysis of the mRNA profile of PLCE1 knockdown in esophageal squamous cell carcinoma

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Abstract. The authors previously reported that Phospholipase C epsilon 1 (PLCE1) exacerbated esophageal squamous cell carcinoma (ESCC), however, the underlying mechanism remains to be fully elucidated. The present study aimed to identify key differentially expressed genes (DEGs) and signaling pathways regulated by PLCE1 in ESCC. EC9706 and Eca109 cell lines were transfected with the specific small interfering (si) RNA of PLCE1, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were performed to detect the expression levels of PLCE1, and subsequently, mRNA array and multiple bioinformatics analysis were conducted. RT-qPCR was used to verify gene expression array results. The findings of the present study indicated that PLCE1 mRNA and protein expression were significantly suppressed (P<0.05) in the PLCE1 siRNA-treated cells. In addition, a total of 223 DEGs with >2-fold alterations were screened between the PLCE1 siRNA-treated cells, including 168 upregulated and 53 downregulated DEGs. In particular, inflammation or immune-associated molecules, including Toll-like receptor (TLR)-4 interleukin-6, -8 and chemokine C-X-C motif ligand 2 were significantly increased following PLCE1 knockdown. Furthermore, Gene Ontology enrichment revealed terms associated with cell proliferation, differentiation, apoptosis, signal transduction, invasion and metastasis, which may potentially be associated with PLCE1 function. Kyoto Encyclopedia of Genes and Genomes pathway analysis demonstrated 46 pathways were disturbed by DEGs, including focal adhesion, mitogen activated protein kinase, TLR, p53 and janus kinase/signal transducer and activator of transcription signaling pathways. The RT-qPCR results for validation of the selected DEGs were consistent with that of the microarray data. Overall, the results of the multiple bioinformatic analysis contributes to a systematic understanding of the roles of PLCE1 in ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC), which ranks the 6th in mortality and the 7th in morbidity in China, is one of the most prevalent malignant neoplasm around the world (1). Low intake of fruits and vegetables, poor nutritional status, smoking, and drinking all contribute to the etiology of ESCC (2). Despite improvements in ESCC treatments, the five-year survival rate of patients with ESCC is still low, because of poor understanding of the molecular pathogenesis of ESCC and infrequent early-stage diagnosis (3,4). Although studies have indicated that some oncogenes and tumor suppressor genes are involved in the progression of esophageal cancer including Phospholipase C epsilon 1 (PLCE1), the molecular mechanisms are still poorly known. Therefore, it is imperative to study the functioning of genes that could be involved in the mechanism of the occurrence and development of ESCC.

Recently, a novel ESCC susceptibility loci located in chromosome10q23 in PLCE1 gene was identified by three genome-wide association studies (GWAS) in Chinese Han populations (5-7). Accumulated evidences have showed PLCE1 plays crucial roles in types of several cancers, such as head and neck (8), bladder (9-11), gastric (12,13), skin (14), prostate (15). Our previous studies revealed increased of PLCE1 expression was significantly associated with lymph node metastasis and advanced TNM stage of Kazakh ESCC, which implicated that PLCE1 could be a novel biomarker for patients of Kazakh ESCC (16). Afterwards, we have stably knocked down PLCE1 by siRNA in ESCC cell lines Eca109 and EC9706, knockdown of PLCE1 resulted in an increase of the apoptosis, a decrease in cell proliferation as well as migration and invasion, and an inhibition of the formation of lamellipodia and filopodia of F-actin in vitro, the effect of PLCE1 on cell migration, invasiveness, and motility is correlated with epithelial-mesenchymal transition (EMT) and cytoskeleton dynamics (4). These results suggested that PLCE1 might play important roles in oncogenesis and progression of ESCC.
Therefore, further research must be carried out to understand the exact role of PLCE1 in ESCC. To better understand the molecular mechanism of PLCE1, the mRNA expression profile of PLCE1 knockdown was analyzed by the Affymetrix Gene Chip Human genome arrays. Nevertheless, a comprehensive analysis of the knockdown of PLCE1 has not been reported for microarray-based techniques in previous studies.

In this study, we explored the functional roles of PLCE1 on downstream genes and signaling pathways in ESCC. Hundreds of differentially expressed genes (DEGs), especially inflammation or immune-related genes, were identified in PLCE1-siRNA-treated cells compared with the control cells. Based on Gene Ontology enrichment and KEGG pathway analysis, we found that PLCE1 knockdown could impact a number of genes involved in proliferation, apoptosis, invasion and metastasis of tumor cells, et al and pathways including MAPK, Toll-like receptor, p33, Focal adhesion, et al. To the best of our knowledge, this is the first paper to perform a systematic analysis of how PLCE1 can influence DEGs and signal pathways in ESCC by microarray-based techniques.

Materials and methods

Cell culture and transfections. Esophageal cancer cell lines (Eca109 and EC9706) were purchased from Institute of Biochemistry and cell biology, Chinese Academy of Sciences (Shanghai, China) Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) at 37˚C under 5% CO2. The transfection of RNA was in the case of the final concentration of 5 nM with HiPerFect transfection reagents (Qiagen, Hilden, Germany) in serum-free conditions. The target sequences for PLCE1-siRNA is 5'-AGC GUU GGU CCAUGCUUAATT-3'.

The DEGs. Raw data were normalized by MAS 3.0 algorithm. The main criterion for selection of DEGs between the two groups of samples being compared: i) q-value ≤ 5% (t-test); ii) fold change ≥2 and ≤0.5; given that there are three types of call values of each detected probe, at lowest one group among the values of three repetition should be non-A values. The expression level of the appropriate gene was the tallest number of P signs, for those genes including a lot of probes. A scatter plot was visualized to evaluate variations from chip to chip.

Gene ontology and pathway enrichment analyses. Molecular annotation system (MAS3.0) was used for gene ontology (GO) enrichment analysis. The 'conditional' option was designed to correct, while 'p value Cutoff' was designed to 0.05. For those GO terms including at least one DEG, a false discovery rate (FDR) was performed by the Benjamini-Hochberg method. FDR was set to 0.1 (cutoff: 0.1).

Pathway enrichment analysis was used to perform via the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/html) database. The KEGG maps of biological functions associated with DEGs were identified. The P-value of the enrichment of DEGs in different pathways was reckoned by the super-geometric test, the significance threshold of false discovery rate (FDR) was also designed to 0.1. Verification for gene expression using quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from ESCC cell lines with mRNA Extraction kit (Qiagen) according to the standard protocol. The cDNA of mRNA was synthesized with One Step PrimeScript mRNA cDNA Synthesis kit (Qiagen). All qRT-PCR data was standardized to β-actin expression. An ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to detect the response. Amplification occurred at an initial denaturation cycle of 95˚C for 5 min followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec. All experiments were performed in triplicate at least three times. The 2-ΔΔCq method was used to quantify the results. The primers used have been listed in Table I.

Western blot analysis. Transfected cells were lysed using RIPA buffer supplemented with Protease Inhibitor (PMSF). Protein was separated by SDS-PAGE, transferred to the PVDF membrane, and prevented by blocking with 0.1% Tween-20 in TBST including 5% nonfat milk for 2 h at room temperature, then, membranes were incubated at 4˚C overnight with primary antibodies against PLCE1 (1:200; rabbit. no. sc-368932; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and β-Actin (1:500; rabbit. no. PR-0255; ZSGB-BIO, Beijing, China), and then incubated with secondary antibodies (1:10,000; rabbit. no. zf-0311; ZSGB-BIO) for 2 h at 37˚C. After washing, membranes were exposed in the darkroom. Finally, the objective bands were analyzed with the ECL procedure.

Statistics. Statistical analysis was carried out by Graphpad Prism 5.0e Software. Results were expressed as mean ± standard deviation comparison between two groups. Differences between means were investigated by paired t-test within two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Si-PLCE1 inhibited PLCE1 mRNA and protein expression. To determine the oncogenic function of PLCE1, si-PLCE1 was used to interfere with PLCE1 expression in Eca109 and EC9706 cells. The endogenous expression of PLCE1 was detected by qRT-PCR and western blot analysis after transfection. The expression of PLCE1 at both mRNA and protein levels was significantly reduced in the PLCE1 knockdown cells, compared with control groups (Fig. 1). These results indicated that the expression levels of PLCE1 mRNA and protein were markedly silenced after transfection in Eca109 and EC9706 cells.

Analysis of the DEGs. To explore the influence of PLCE1 on downstream genes, the mRNA expression profile was analyzed using the Affymetrix GeneChip Human genome 3, IVT arrays which contain 49294 genes in Eca109 and EC9706 transfected with PLCE1 siRNA compared to control groups.

Results compared with control groups, a total of 223 DEGs were identified by greater than 2.0-fold, of these, 168 DEGs were robustly upregulated and 55 DEGs were robustly downregulated. Primitively, the significantly increased genes were identified, such as DUSP5, IL8, IER3, IL6 and so on, while...
significantly downregulated genes including \textit{TMEM30A}, \textit{SNTB2}, \textit{TMEM106B} and so on (Table II). Interestingly, the expression of inflammatory factors was significantly increased after \textit{PLCE1} gene silencing, such as \textit{IL-8}, \textit{IL-6}, \textit{CXCR4} and so on. Contrary to popular belief, inflammation was regarded as a promoter of tumor formation and metastasis. The results of our study suggested that \textit{PLCE1} might inhibit inflammatory factors expression. A two-dimensional rectangular coordinate plane was shown a scatter plot (Fig. 2). Collectively, \textit{PLCE1} might have a significant impact on expressions of \textit{PLCE1}-modulated downstream genes. Accordingly, a lot more researches are needed to figure out the possible relationship between these genes and \textit{PLCE1}.

\textbf{GO enrichment analyses.} In order to have a command of the functional categories of that DEGs involved in and to find

Table I. The primers used for Q-RT-PCR.

| Gene name | Primers | Annealing temperature (°C) | The length of product (bp) |
|-----------|---------|----------------------------|---------------------------|
| \textit{PLCE1} | F:GAGCTGCAATCGAAGTCTGG  
R:AAGGCCTTCTGTGAGTTCCTC | 60 | 192 |
| \textit{IL-1a} | F:ACGACTGGTTCTCAATCAGG  
R:CTGCATGACTCGGCTTTATCA | 60 | 142 |
| \textit{IL-1b} | F:CCAGGATGAGGACCTGAGAA  
R:CGAGGATTTGCTGTTGTTCA | 60 | 149 |
| \textit{CXCII} | F:CCCCAAACGGAAGTCATAGC  
R:GATTGTACTGTTCAGCATCTTT | 58 | 109 |
| \textit{CXCL2} | F:CGAAGTCATAGCACCACACTCAAG  
R:CTTCTGAGTCTGATGAGTGC | 58 | 116 |
| \textit{CCL2} | F:GCACCTTCGCCCTCCAGCATGA  
R:CAGCAGGTGACTGGGCAATTGA | 50 | 121 |
| \textit{CCL20} | F:TGCTGTACCAGAGATTTGGCTC  
R:CGCACACAGAACCATTTCCTTT | 58 | 217 |
| \textit{β-actin} | F:TTGAAGTGTACGGCAGATCCGC  
R:GCCAATCTCATCTTGTTTGTGCGC | 60 | 356 |

F, forward; R, reverse.
Table II. Differentially expressed genes in PLCE1 siRNA-Treated esophageal squamous cell carcinoma cells, compared with control cells.

A. Upregulated genes

| Gene symbol | Fold change | Gene symbol | Fold change | Gene symbol | Fold change |
|-------------|-------------|-------------|-------------|-------------|-------------|
| IL8         | 22.624      | HSPA5       | 3.007       | TNRC6A      | 2.27        |
| CXCL2       | 10.224      | SAT1        | 3           | IFI16       | 2.26        |
| IFI27       | 10.111      | CLDN1       | 2.966       | HSPA5       | 2.26        |
| IFIT2       | 8.896       | PRIC285     | 2.929       | CXC4        | 2.252       |
| CXCL2       | 8.1833      | PLSCR1      | 2.925       | SOD2        | 2.249       |
| OASL        | 7.9428      | PHLD1A      | 2.908       | JUN         | 2.246       |
| 11762907_at | 7.457       | RELB        | 2.908       | DUSP6       | 2.242       |
| 11760093_at | 7.012       | NFKBIZ      | 2.879       | FNDC3A      | 2.241       |
| IFIT1       | 6.821       | NTSE        | 2.808       | GDF15       | 2.238       |
| DDI3T       | 6.788       | STC2        | 2.802       | SAMD9       | 2.232       |
| DDX58       | 6.5573      | OAS1        | 2.734       | HIF1A       | 2.23        |
| DDI3T       | 6.071       | FNDC3A      | 2.715       | PLSCR1      | 2.225       |
| IFIT3       | 5.934       | SERPINE2    | 2.691       | TNRC6A      | 2.214       |
| IL6         | 5.899       | F3          | 2.663       | ZC3HAV1     | 2.214       |
| TRAC         | 5.221       | ZC3H12C     | 2.663       | IFITM1      | 2.202       |
| GDF15       | 5.007       | SAT1        | 2.613       | PLSCR1      | 2.198       |
| PTGS2       | 5.005       | ADM         | 2.61        | ADRB2       | 2.197       |
| ISG20       | 4.985       | IRF7        | 2.596       | CDKN1A      | 2.194       |
| TNFRSF21    | 4.789       | ABCD3       | 2.592       | SPINK1      | 2.193       |
| IRF9        | 4.719       | OAS2        | 2.59        | CLEC2B      | 2.181       |
| 11761908_at | 4.698       | ADM         | 2.562       | PMAIP1      | 2.157       |
| GDF15       | 4.418       | FNDC3A      | 2.522       | ZC3H12C     | 2.156       |
| PTGS2       | 4.31        | ITG2A       | 2.506       | FGFR2       | 2.143       |
| C5orf41     | 4.295       | IRF1        | 2.503       | SIRPA1L2    | 2.136       |
| ISG15       | 4.122       | PARP9       | 2.503       | FICD        | 2.129       |
| GDF15       | 4.091       | PLSCR1      | 2.501       | DUSP6       | 2.114       |
| TNFRSF21    | 4.05        | DNAJB9      | 2.5         | HSPA5       | 2.112       |
| STC2        | 4.024       | ICAM1       | 2.495       | RSP03       | 2.108       |
| DUSP5       | 4.003       | MAFF        | 2.482       | ANGPTL4     | 2.107       |
| TNFAP3      | 3.996       | FAM3        | 2.475       | SAMHD1      | 2.105       |
| SERPINE2    | 3.81        | SAA1/SAA2   | 2.47        | PPP1R15A    | 2.103       |
| PLSCR1      | 3.805       | STX11       | 2.454       | B3GNTS      | 2.086       |
| RND3        | 3.796       | CDKN1A      | 2.441       | FNDC3B      | 2.078       |
| TNFAP3      | 3.743       | DUSP6       | 2.44        | USP18       | 2.076       |
| AMIGO2      | 3.664       | ICAM1       | 2.427       | RND3        | 2.069       |
| PTGS2       | 3.533       | CXCR4       | 2.422       | FNDC3B      | 2.068       |
| SERPINE2    | 3.492       | STAT1       | 2.415       | ELL2        | 2.062       |
| HSPA5       | 3.483       | PMAIP1      | 2.407       | JAG1        | 2.049       |
| IFI6        | 3.462       | PTGER4      | 2.386       | MCL1        | 2.048       |
| FOS         | 3.427       | HERC5       | 2.385       | TLR4        | 2.048       |
| FOSL1       | 3.419       | FOSL1       | 2.378       | DRAM1       | 2.044       |
| HSPA5       | 3.417       | BIRC3       | 2.347       | OAS1        | 2.043       |
| SAMD9L      | 3.409       | BIRC3       | 2.346       | SIPA1L2     | 2.038       |
| ATF3        | 3.387       | FGF2        | 2.346       | OTUD1       | 2.037       |
| C5orf41     | 3.231       | PLSCR1      | 2.345       | GFTP1       | 2.032       |
| FOSL1       | 3.196       | VEGFC       | 2.336       | OSMR        | 2.026       |
| HIVEP2      | 3.148       | OAS1        | 2.335       | GADD45A     | 2.023       |
| BIRC3       | 3.131       | JUN         | 2.33        | ETV4        | 2.021       |
| CLDN1       | 3.128       | HRASLS2     | 2.317       | PLAUR       | 2.019       |
out which GO terms can be strongly enriched or significantly associated with our selected genes, GO term enrichment analysis was performed by MAS3.0. As is shown in Fig. 3, these DEGs were sorted and categorized into 26 different functional categories by \textit{PLCE1} knockdown in ECa109 and EC9706 cells. While 442, 178 and 94 significant GO terms for these DEGs were identified in Biological Process, Molecular Function and Cellular Component.

Remarkably, among the Biological Process, predominant terms were identified as follows. Firstly, the downregulated DEGs involved in cell division (GO:0051301), as well as upregulated DEG linked to cell cycle arrest (GO:0007050) and negative regulation of cell proliferation (GO:0008285) were represented, which revealed the inhibition of the \textit{PLCE1}-mediated cell might block cell proliferation via these DEGs. Secondly, upregulated DEGs were enriched in anti-apoptosis (GO:0006916), negative regulation of apoptosis (GO:0008285), indicating these terms might have direct or immediate adverse effect on apoptosis induction in \textit{PLCE1}-siRNA-treated Eca109 and EC9706 cells. Thirdly, upregulated genes were also enriched in cell-cell signaling (GO:0007165), \textit{G}-protein coupled receptor protein signaling pathway (GO:0007166), which suggested these changes were likely to participate in the oncogenesis and tumor development of ESCC. To our interest, a significant proportion of clusters was enriched in ‘response to virus (GO:0006915)’, ‘immune response (GO:0006955)’, ‘chemotaxis (GO:0006935)’ and ‘inflammatory response (GO:0006954)’ in the biological process category, suggesting

Table II. Continued.

A, Upregulated genes

| Gene symbol | Fold change | Gene symbol | Fold change | Gene symbol | Fold change |
|-------------|-------------|-------------|-------------|-------------|-------------|
| CPA4        | 3.123       | ZFP36       | 2.316       | JUN         | 2.016       |
| FOSL1       | 3.12        | ABCD3       | 2.297       | IRF9        | 2.014       |
| IRAK2       | 3.089       | IFITM1      | 2.294       | DUSP4       | 2.011       |
| IER3        | 3.081       | OAS1        | 2.293       | SERPINE1    | 2.009       |
| GABBR1      | 3.052       | CXCR4       | 2.293       | KRT34       | 2.005       |
| RHEBL1      | 3.032       | SAMHD1      | 2.281       | SLC6A6      | 2.0008      |
| SOD2        | 3.013       | DUSP6       | 2.271       | HERPUD1     | 2.0004      |

B, Downregulated genes

| Gene symbol | Fold change | Gene symbol | Fold change | Gene symbol | Fold change |
|-------------|-------------|-------------|-------------|-------------|-------------|
| TMEM30A     | 0.255       | C5orf53     | 0.418       | SEPP1       | 0.468       |
| TMEM30A     | 0.259       | SUV39H1     | 0.42        | NEUR1LB     | 0.474       |
| SN7B2       | 0.311       | NCRNA00201  | 0.426       | MOBKL2B     | 0.476       |
| C2orf45     | 0.345       | GNAI1       | 0.43        | LOXL1       | 0.477       |
| IGSF8       | 0.348       | VEPH1       | 0.439       | SUV39H1     | 0.478       |
| STYX        | 0.357       | CAV1        | 0.446       | CHST13      | 0.48        |
| C2orf45     | 0.36        | C8orf3      | 0.453       | PSKH1       | 0.481       |
| STYX        | 0.371       | MOBKL2B     | 0.454       | TMEM106B    | 0.482       |
| DAZAP1      | 0.374       | CLIC3       | 0.455       | ZNF362      | 0.483       |
| C7orf41     | 0.385       | METTL7A     | 0.456       | BMP8B       | 0.483       |
| FAM107B     | 0.398       | SORBS2      | 0.458       | SGOL2       | 0.486       |
| CCNF        | 0.399       | LMO1        | 0.459       | TMEM106B    | 0.486       |
| FAM107B     | 0.403       | CBX5        | 0.46        | FAM47E      | 0.488       |
| HNRRNPU     | 0.404       | SORBS2      | 0.463       | CBX5        | 0.49        |
| SN7B2       | 0.406       | CBX5        | 0.463       | RNF34       | 0.491       |
| GNAI1       | 0.408       | SREK1P1     | 0.464       | AMY1A       | 0.492       |
| ALPI        | 0.409       | SAMD11      | 0.465       | EXD3        | 0.494       |
| DPH3        | 0.41        | MGD3        | 0.468       | PABPC4L     | 0.495       |
|             |             |             |             | SUV39H1     | 0.497       |
that the changes related to these terms might take place during the genesis of ESCC.

Besides, a few terms connected to cytoskeleton organization were identified from Molecular Function and Cellular Component, including actin binding (GO:0003779), actin cytoskeleton (GO:0015629), focal adhesion (GO:0005925), integrin complex (GO:0008305), indicated that PLCE1 might exert significant influence in the metastasis, invasion and mobility of ESCC through the functions of genes implicated in these terms.

KEGG pathway enrichment analyses. To find out how the DEGs from PLCE1 knockdown influence process of cell biological function, KEGG pathway enrichment analysis was carried out to identify relevant pathways. Based on the KEGG pathway analyses, these DEGs were enriched in 46 pathways which changed significantly (q-value <0.05), containing MAPK, Toll-like receptor, p53, JAK-STAT signaling pathway, small cell lung cancer, renal cell carcinoma, bladder cancer, prostate cancer, cytokine-cytokine receptor interaction, focal adhesion, ECM-receptor interaction concluding consist of ITGA2, Starch and sucrose metabolism consisting of AMY1A, AMY1B, AMY1B, AMY1C, AMY2A, AMY2B, AMY2B, gluta-mate metabolism covering GFPT1, Nitrogen metabolism extending to NT5E and so on (Table III). The majority of these pathways were involved in cell biological function, including proliferation, invasiveness and apoptosis. Nevertheless, compared with the other signaling pathway, the majority of DEGs were significantly enriched in MAPK, JAK-STAT, P53, TLRs signaling pathways and the Focal adhesion. We should therefore concentrate our interest on the genes associated with the above pathways. The DEGs involved in the above signaling pathways are represented. There were alterations involved in the focal adhesion including 5 upregulated and 1 downregulated DEGs. Among the MAPK signaling pathways -related genes, expression changes mainly occurred in 10 genes, including DUSP6, RELB, FGF2, FOS, DUSP4, DDIT3 (GADD153), DUSP5, and JUN between the PLCE1 siRNA and control group. The data of gene chip shown 4 upregulated genes (PMA1PI, SERA1PI (PAI-1), CDKN1A (P21), GADD45) in P53 signaling pathway, deregulated with a >2-fold difference following PLCE1 siRNA transfection in Eca109 and EC9706 cells. There were 7 upregulated genes (STAT1, FOS, TLR4, IL6, IL-8) with >2-fold changes between the treatment and un-treatment cell involved in TLRs signaling pathways. Thus, our results indicated a strong interaction between PLCE1 and these pathways that DEGs were involved in.

Conformation of genes associated with inflammatory factors by qRT-PCR after PLCE1-siRNA transfection. To approve or confute the results of our mRNA microarray analysis, qRT-PCR analysis was performed to evaluate mRNA levels of six pivotal DEGs selected in EC9706 and ECa109 trans-fected with PLCE1-siRNA. As expected, the results were consistent with the results of microarray findings that implicated involvement of IL-1a, IL-1b, CXCL-1, CXCL-2, CCI-2, CCL-20 genes. Compared with control group, by Q-RT-PCR analysis, all 6 genes were upregulated by PLCE1-siRNA treatment of EC9706 and ECa109 cells (Fig. 4). The results were corresponded to that of microarray data, which bore out the reliability of the results.

Discussion

ESCC, which is the fourth leading cause of cancer death, is one of the most aggressively malignant tumors in China (17). It was reported that PLCE1 was a susceptibility gene in ESCC. Although PLCE1 has been extensively studied, it was not consistent with its
| Pathway                                                   | Count | P-value     | Gene                                                                 |
|-----------------------------------------------------------|-------|-------------|----------------------------------------------------------------------|
| MAPK signaling pathway                                    | 10    | 2.03E-09    | DUSP6; RELB; FGF2; FGF13; FOS; DUSP4; DDIT3; GADD45A; DUSP5; JUN     |
| Toll-like receptor signaling pathway                      | 7     | 7.46E-09    | STAT1; FOS; TLR4; IL6; JUN; IL8; IRF7                               |
| Starch and sucrose metabolism                             | 5     | 2.07E-07    | AMY1A; AMY1A; AMY1B; AMY1C; AMY2A; AMY2A; AMY2B                     |
| Cytokine-cytokine receptor interaction                    | 7     | 4.52E-06    | CXCR4; TNFRSF21; OSMR; CXCL2; IL6; IL8; VEGFC                      |
| Focal adhesion                                            | 6     | 1.33E-05    | BIRC3; ITGA2; CAV1; PAK3; JUN; VEGFC                              |
| p53 signaling pathway                                     | 4     | 2.78E-05    | PMAIP1; SERPINE1; CDKN1A; GADD45A                                 |
| Renal cell carcinoma                                      | 4     | 3.11E-05    | HIF1A; PAK3; JUN; VEGFC                                           |
| Bladder cancer                                            | 3     | 0.00016     | CDKN1A; IL8; VEGFC                                               |
| Jak-STAT signaling pathway                                | 4     | 0.00063     | STAT1; OSMR; IRF9; IL6                                           |
| Complement and coagulation cascades                       | 3     | 0.0007      | PLAUR; SERPINE1; F3                                           |
| Melanoma                                                  | 3     | 0.00076     | FGF2; FGF13; CDKN1A                                            |
| B cell receptor signaling pathway                         | 3     | 0.00089     | FOS; IFITM1; JUN                                               |
| Small cell lung cancer                                    | 3     | 0.00132     | BIRC3; PTGS2; ITGA2                                          |
| ErbB signaling pathway                                    | 3     | 0.00137     | CDKN1A; PAK3; JUN                                        |
| Regulation of actin cytoskeleton                          | 4     | 0.00221     | FGF2; FGF13; ITGA2; PAK3                                      |
| T cell receptor signaling pathway                         | 3     | 0.0026      | FOS; PAK3; JUN                                                      |
| Leukocyte transendothelial migration                      | 3     | 0.00349     | CXCR4; CLDN1; ICAM1                                         |
| Cell adhesion molecules (CAMs)                            | 3     | 0.00465     | CLDN1; ICAM1; CNTN1                                           |
| mTOR signaling pathway                                    | 2     | 0.00751     | HIF1A; VEGFC                                                      |
| Pathogenic Escherichia coli infection - EHEC              | 2     | 0.00837     | CLDN1; TLR4                                                     |
| Pathogenic Escherichia coli infection - EPEC              | 2     | 0.00837     | CLDN1; TLR4                                                     |
| Epithelial cell signaling in Helicobacter pylori infection | 2     | 0.01328     | JUN; IL8                                                        |
| Pancreatic cancer                                         | 2     | 0.01438     | STAT1; VEGFC                                                    |
| Colorectal cancer                                         | 2     | 0.01874     | FOS; JUN                                                        |
| Hematopoietic cell lineage                                | 2     | 0.02002     | ITGA2; IL6                                                       |
| Apoptosis                                                 | 2     | 0.02089     | BIRC3; IRAK2                                                    |
| Neuroactive ligand-receptor interaction                   | 3     | 0.02635     | GABBR1; PTGER4; ADRB2                                          |
| GnRH signaling pathway                                    | 2     | 0.02939     | GNA11; JUN                                                      |

KEGG, Kyoto encyclopedia of genes and genomes; DEG, differentially expressed genes.
role in cancer. Previous findings demonstrated PLCE1 has been identified as a tumor suppressor gene of colorectal tumor (18). However, PLCE1 has also acted as a novel oncogene in a variety of human tumors, such as bladder cancer (9) and head and neck cancer (8). Hence, PLCE1 promoting or inhibiting the occurrence and development of tumor is still to be further studied. Our previous studies showed increased expression of PLCE1 could be used as a potential biomarker for the early diagnosis and poor prognosis of ESCC (16). Furthermore, we also found PLCE1 knockdown effectively inhibited cell growth/proliferation and increased apoptosis as well as reduced the invasion and migration in ESCC (4). Our results demonstrated that PLCE1 could play a tumor-oncogenic function in ESCC. Thus, it would be of great use to have systematic understanding of the functions of PLCE1. Here we presented a comprehensive analysis of mRNA profile of PLCE1 knockdown in ESCC cells by multiple bioinformatic analyses.

In the present study, a total of 223 DEGs, including 168 upregulated and 55 downregulated DEGs, were identified. In order to have a command of the functions of these DEGs, the DEGs were analyzed by GO enrichment tools. In the present study, these upregulated DEGs were significantly enriched in cell cycle arrest (PPP1R15A, DIT3, CDKN1A, GADD45A, IL8), negative regulation of cell proliferation (PTGS2, FGF2, IL6, CDKN1A, IFITM1, IL8), apoptosis (FGF2, CXCR4, TNFRSF21, PPP1R15A, RNF34, GADD45A, TNFAIP3, IER3, PHLD1A), cell-cell signaling (STC2, FGF2, ADM, GDF15, ISG15, IL8), cell adhesion (CLDN1, AMIG02, RND3, ITGA2, F3), these results supported such an assumption that oncogenic PLCE1 was involved in esophageal proliferation, apoptosis and metastasis. Besides that, KEGG pathway enrichment analysis was carried out to identify relevant pathways, these DEGs were identified significantly enriched in 46 pathways. Among them, DEGs are closely linked to a variety of different signaling networks, including the MAPK, p53, JAK-STAT, Toll-like receptor signaling pathways. A following discussion focuses mainly on the alteration of genes involved primarily in MAPK, p53, Toll-like receptor signaling pathways.

In the MAPK signaling pathway, several genes from the MAPK signaling pathway are altered in cells of PLCE1 downregulation, including 10 upregulated genes (DUSP6, DUSP4, DUSP5, RELB, FGF2, FGF13, FOS, DIT3, GADD45A, JUN). Mitogen-activated protein kinase (MAPK) pathways, which has extracellular regulated kinases 1 and 2 (ERK1/2), c-Jun-N-terminal and p38, regulate many cellular functions including cell proliferation, differentiation, migration and apoptosis. Study showed that CDC25 homology domain of PLCE exhibited GEF activity toward Rap1, transiting from the Rap1.GDP-bound state to the Rap1.GTP-bound state facilitated by GEF, leading to the accumulation of the formation of Rap1GTP, thereby, which in turn stimulates B-Raf/MEK/ERK pathways within the cell, in addition, activation of Rap2B stimulates PLCE1, then activating H-Ras results in cascade response corresponding to MAPK, finally MAPK was activated, and promoting cell proliferation. In addition, our previous study has shown that ERK1 protein expression was significantly increased in the ESCC tissues (19), thus it can be seen that PLCE1 might promote the incidence of esophageal carcinoma indirectly via MAPK/ERK1/2 pathways. Fibroblast growth factor 2 (FGF2) activated a series of intracellular signal transduction pathways by tyrosine kinase receptors (FGFR) to form a complete pathway: FGF2/MAPK/ERK1/2 (20,21), which finally induces various cellular activities, including mitosis, differentiation, proliferation, and cell migration (22,23). However, DUSP6, DUSP5 and DUSP4, belonging to dual specificity phosphatase (DUSP) family, limits over-activation of the FGFs/MAPK/ERK1/2 signal transduction pathways in a negative feedback manner (24). This implies that the PLCE1 siRNA-induced apoptosis and proliferative inhibition in ESCC are related to DUSPs/FGFs/MAPK/ERK1/2 signal pathways. These findings provide a novel mentality to study the pathogenesis of ESCC in MAPK signaling pathway.

As known, p53 is one of the most important tumor suppressor genes in cancer-associated diseases and plays a crucial role in regulating cell cycle arrest, apoptosis and senescence (25), recent study also revealed p53 is involved in PLCE1-knockdown induced apoptosis in Esophageal Cancer cells (26). Our results demonstrated 2 key upregulated components CDKN1A (P21) and GADD45A involved in the p53 signaling pathway were upregulated in PLCE1 siRNA treated cells. Following the downstream target of p53, p21 and GADD45A are activated and block the cell cycle at the G1 and G2 phases (27,28). This result suggests that PLCE1 inhibition may lead to an increase in p53 function by upregulating CDKN1A (P21) and GADD45A,
and that a new PLCE1/p53/p21 pathway is involved in PLCE1-knockdown induced apoptosis and cell cycle arrest in ESCC. In addition, cellular senescence is primarily regulated by p53/p21 and p16/pRb pathways, where the p53/p21 pathway mediates the replicative senescence and plays crucial roles in DNA damage response (29). Therefore, PLCE1 suppression might have an effect on the p53/p21 signaling pathway through p53 and p21 upregulation, leading to proliferation inhibition and inducing cell senescence and in Eca109 and EC9706 cells.

In this study, we have shown that inflammation or immune-related molecules (TLR4, IL-8, IL-6, CXCL2) were significantly increased after PLCE1 suppression, and the results by qRT-PCR analysis were in agreement with that of microarray data. However, these results did not agree with recent reports in which PLCE was shown to enhance tumor development in many human cancers (30-32), by increasing the production of inflammatory cytokines in local tissues, obviously, our results suggested that PLCE1 might be involved a yet unknown signaling pathway linking tumor promotion and cytokines. Interestingly, it was reported that the expression of cytokines IL-1α, IL-6, and IL-8 initiated and supported cellular senescence (33). Similar report also showed that IL-1α activated tumor-suppressive pathways to maintain senescent growth (34). Nevertheless, cellular senescence restricts unlimited cell proliferation and plays an essential role in tumor suppression. Therefore, we investigate whether PLCE1 could enhance carcinogenesis and progression of ESCC via loss of cytokines-supported senescence. In addition, it was well recognized that toll-like receptor 4 (TLR4), an intensively studied member in the TLR family, results in transcriptional activation of pro-inflammatory genes including cytokines (IL-1α, IL-6, CXCL-2) (35). Importantly, TLR4 activation inhibited hepatocellular carcinogenesis due to immune networks (IL-1α/β, IL-6, CXCL-2) triggering cellular senescence (36). These results taken together suggested PLCE1 could decrease cellular senescence via inhibition of the TLR4 signaling pathway, which contributed to the development of ESCC. In future studies, it will be worthwhile to determine whether PLCE1 has a role in esophageal carcinoma by loss of TLR4-mediated immunity supporting senescence.

In summary, the comprehensive understanding the role of PLCE1 in ESCC was obtained by bioinformatics workflow of mRNA profile after PLCE1 knockdown by multiple bioinformatics analysis. A total of 223 DEGs, 168 DEGs upregulated and 55 downregulated DEGs, were identified in PLCE1-siRNA-treated cells compared with the control cells. Alterations genes in the Focal adhesion, MAPK, P53 and TLRs signaling pathways may be important during PLCE1 siRNA-induced apoptosis, and proliferation, invasive and migrant inhibition of Eca109 and EC9706 cells. These findings provide information useful for combination targeted therapy in PLCE1-relevant neoplasm. We need to characterize DEGs and identify the exact molecular mechanism PLCE1 involved in proliferation, apoptosis, invasion and metastasis.

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