JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Bioinformatics analysis of microarray profiling identifies the mechanism of focal adhesion kinase signalling pathway in proliferation and apoptosis of breast cancer cells modulated by green tea polyphenol epigallocatechin 3-gallate

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Keywords
bioinformatics analysis; breast cancer; epigallocatechin 3-gallate; focal adhesion kinase signalling pathway

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Received June 1, 2018
Accepted August 10, 2018

doi: 10.1111/jphp.13010

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Abstract

Objectives This study aimed to investigate potential gene and signal pathway associated with tumour progression.

Methods Related microarray data set of breast cancer was obtained from Gene Expression Omnibus database, and differential-expressed genes (DEGs) between two control samples and two treated samples were analysed using statistical software R. We collected 50 epigallocatechin-3-gallate(EGCG)-related genes and 119 breast cancer-related genes to create a knowledge base for following pathway analysis.

Key findings A total of 502 mRNAs were identified as DEGs based on microarray analysis. Upregulated DEGs mainly enriched in nuclear nucleosome, cell adhesion, DNA packaging complex, Wnt-activated receptor activity, *etc.*, while the downregulated DEGs significantly enriched in ncRNA processing, mitotic nuclear division, DNA helicase activity, *etc.* DEGs mostly enriched in gap junction, cell cycle, oxidative phosphorylation, focal adhesion, *etc.* EGCG suppressed FAK signalling pathway. Furthermore, EGCG could inhibit breast cancer cell proliferation and promote apoptosis by modulating CCND1.

Conclusions Epigallocatechin 3-gallate might exert influence on breast cancer progression through inhibiting focal adhesion kinase (FAK) signalling pathway.

Introduction

Breast cancer is one of the most prevalent malignancies and a main culprit of cancer-related death in female population worldwide.[1] Approximately 13% of women in the United States are likely to develop invasive breast cancer, and the morbidity rate of breast cancer among women in the UK has risen by 6% in recent years.[2] Both inherited and environmental factors lead to the high incidence of breast cancer. Researches in the last several decades have illuminated the roles of multiple oncogenes, tumour inhibitors and their associated signal transduction pathways in the progression of breast cancer.[3–5] It has been reported that more than 70% of sporadic breast cancers are attributable to long-term exposure to environmental factors, such as chemical carcinogens.[6] Over 200 chemical mammary carcinogens have been experimentally detected to bring about breast cancer progression and tumorigenesis.[7–9]

Green tea, rich in powerful antioxidant, is a kind of popular beverage consumed around the world. Of all the antioxidant compounds found in green tea, the major constituents are polyphenols, including phenolic acids and catechins. Catechins derived from green tea belong to the family of flavonoids that are powerful antioxidants and free iron scavengers.[10] It has been generally recognized that much of cancer chemopreventive effects of green tea are mediated by its catechins. Research showed a typical cup of green tea contains 250–375 mg catechins, accounting for 10–15% of the dry weight of the leaves.[11] The major constituent catechins in green tea incorporate (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epicatechin (EC). Heinrich *et al.*[12] detected there were about 1402 mg/l catechins in green tea, and the concentration of EGCG was approximately 980 mg/l while EGC was about 6.0 mg/l in green tea. Some studies compiled the statistics that intake of EGCG with green tea infusions or beverages is up to about 450 mg EGCG/person/day in Europe and higher in Asia.[13]

As for the link between green tea consumption and cancer risk, there was disagreement. Hou *et al.* analysed researches on gastric cancer; the relative risks or odds ratio of gastric cancer for the highest level of green tea consumption was compared, and seven studies suggested no association, eight researches suggested an inverse association, and one suggested a positive association.[14] Cumulative epidemiologic evidence revealed that green tea consumption is not associated with pancreatic cancer.[15] A report from Shanghai Men's healthy study exhibited the colorectal cancer risk decreased as the amount of green tea consumption increased.[16]

Butler *et al.* integrated some related literatures and performed META analysis of them. The result revealed there was significant inverse association between green tea intake and risk of ovarian/endometrial cancer, but more data were needed to evaluate whether green tea can reduce risk of human papillomavirus-related cancers.[17] On the contrary, Wu *et al.*[18] found that significant breast cancer risk reduction with regular green tea intake after adjustment for soy and other potential confounding factors. Taken together, whether catechin in green tea can inhibit breast cancer remains to be determined, there are few studies on the regulatory effects of EGCG and CCND1 on cancer, further studies are needed.

Among the catechins found in green tea, the most abundant and most biologically active is epigallocatechin-3-gallate (EGCG), accounting for 50–80% of the total tea catechins.[11] Therefore, EGCG plays an important role in the effects of catechins on biological processes. EGCG is the most abundant and biologically active catechin in green tea.[19] EGCG, as a major catechin, has multiple therapeutic effects including antioxidant, anticancer and immunomodulatory effects.[20,21] Cumulative studies have identified EGCG as a new effective drug with minimal side effects for the treatment of multiple types of cancers.[22] Shankar *et al.*[23] disclosed that EGCG restrained cell growth, invasion, angiogenesis and metastasis in pancreatic cancer. Liang *et al.*[24] reported that EGCG inhibited cell propagation and metastasis in breast cancer by repressing cyclin-dependent protein activity and downregulating VEGF and MMP-9 expressions. Additionally, considerable researches suggested that EGCG can influence cancer initiation and progression through modulating signal transduction pathways or related gene expressions.[25] Hong *et al.*[26] unravelled that epigallocatechin gallate restrained the growth of MDA-MB-231 breast cancer cells via inactivation of the β-catenin signalling pathway. Chung *et al.*[27] uncovered that epigallocatechin gallate inhibited the cancer stem cell phenotype via downregulation of STAT3-NFκB signalling and CD44 expression. Lee *et al.*[28] demonstrated that EGCG could impede cell propagation in human oral squamous cell carcinoma through upregulating *BTG2* via p38 and ERK pathways. Nonetheless, the specific functions and effects of EGCG on breast cancer-related genes and signal transduction pathways remained to be further investigated.

Gene expression profiling is commonly used to detect differentially expressed genes in cancer tissue compared with nontumour tissue.[29] Previous studies using microarray profiling have analysed differentially expressed genes (DEGs) in EGCG-treated cancer cells based on microarray profiling.[30–32] Herein, we identified possible mechanisms of focal adhesion kinase (FAK) signalling pathway by which EGCG could reduce breast cancer risk by means of bioinformatics analysis of microarray profiling. In the present experiments, GSE56245 was downloaded from GEO database and used to identify the DEGs between two control groups and two EGCG-treated groups so as to investigate the underlying molecular mechanisms of breast cancer. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to analyse the differentially expressed genes (DEGs). Furthermore, gene and protein networks were also established by STRING and Cytoscape to investigate the interaction of breast cancer-related genes and KEGG pathways. Moreover, MTT and flow cytometry assays were utilized to examine the influence of EGCG on FAK signalling pathway gene expression and breast cancer cellular events. Our study was based on exploring the effect of EGCG on the progress of breast cancer, and there were few studies on the possible regulation of EGCG on CCND1 and thus on the influence of FAK signalling pathway. Our study was quite novel to research the mechanism combining with this regulatory axis. The results of this study might facilitate the understanding of the mechanisms of FAK signalling pathway involving EGCG-mediated breast cancer cell propagation and apoptosis.

Materials and Methods

Microarray data

Microarray expression profile data set of GSE56245 including two control groups and two EGCG-treated groups was acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The platform for GSE56245 was GPL570 Affymetrix Human Genome U133 Plus 2.0 Array (Santa Clara, CA, USA).

Identification of DEGs

DESeq2 package was used for examination of DEGs between control and EGCG-treated groups according to the instructions. Volcano plot and heatmap were, respectively, drawn using the ggplot2 and pheatmap package. DEGs were identified adjust P value <0.05 and $\log_2$FC (fold change) > 1 of expression level. Twenty DEGs including *CCND1* were selected to plot the pheatmap.

Gene set enrichment analysis (GSEA)

Differences between gene expressions of biological pathways in control and EGCG-treated groups were compared through GSEA 3.0 with reference gene sets from the Molecular Signatures Database (MSigDB) of c2 (KEGG gene sets: c2.cp.kegg.v6.1.symbols.gmt) and c5 (GO gene sets: c5.bp.v6.1.symbols.gmt, c5.bp.v6.1.symbols.gmt, c5.cc.v6.1.symbols.gmt and c5.mf.v6.1.symbols.gmt).[33] The number of permutations was set at 1000, gene set was chosen for permutation type, and metric for ranking genes chose ratio of classes.

Functional annotation and pathway enrichment analysis of DEGs

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v.6.8 (https://david.ncifcrf.gov/) was used to analyse input genes and classify gene functions. To identify the functional annotation of DEGs, we analysed KEGG pathway enrichment and GO terms, while P-value cut-off of <0.05 was deemed statistically significant.

Gene and protein networks and module analysis

EGCG-related genes were obtained from STITCH (http://stitch.embl.de/) which is a database of known and predicted interactions between chemicals and proteins. Breast cancer-related genes were obtained from Disease Gene Search Engine with Evidence Sentences (DigSee) (http://210.107.182.61/geneSearch/). The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/) which provides a significant assessment and integration of gene–gene interactions was used to assess direct and indirect associations of DEGs.

Reagents

Breast cancer cell line MCF7 was purchased from BeNa Culture Collection, stored at −80 °C and upon thawing diluted in fresh cell culture medium immediately before use. EGCG was purchased from Sigma-Aldrich, and the purity of EGCG was over 95%. EGCG was dissolved in sterile cell culture medium at the required concentration immediately before use.

Cell culture

MCF7 cells were cultured in phenol red containing DMEM-F12 supplemented with 10% foetal bovine serum (FBS, HyClone, Invitrogen, Camarillo, CA, USA) and 100 units/ml of penicillin and 100 μg/ml of streptomycin, and cells were incubated at 37 °C with 5% CO_2 in humidified incubator. Cells were treated with 40 μM of EGCG dissolved in ethyl alcohol (EtOH) and incubated further for 24 h before harvesting the cells for total RNA extraction. At the same time, cells that merely treated with EtOH regarded as control groups.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA extraction and quantitative real-time PCR procedures were performed as previously described. Reverse transcription was implemented using Prime Script RT reagent Kit (Takara, Japan) following the protocols of manufacturer. QRT-PCR was conducted using SYBR Prime Script RT-PCR Kits (Takara, Japan) based on the producer's manual. The CCND1 was normalized to GAPDH mRNA through $2^{-\Delta\Delta Ct}$ method. PCR primers were generated as follows: *CCND1* (forward, 5′-CTGTCCCACTCCT ACGATACG-3′; reverse, 5′-CAGCATCTCATAAACAGGT CACTAC-3′); GAPDH (5′-AAGGCTGAGAACGGGAAGC-30 (3F) and 50-GAGGGATCTCGCTCCTGGA-3′).

Cell transfection

For transient transfections, cells were seeded in a 24-well plate. EGCG-treated cells were subsequently transfected with NC, pEGFP-N1-CCND1 and that purchased from GenePharma (Shanghai, China). All transfections were performed with Lipofectamine 3000 following the manual of manufacturer (Invitrogen, Grand Island, NY, USA). Cells

in the NC group were transfected with empty vectors. After post-transfection for 48 h, cells were harvested.

Western blot

Following incubation for 48 h, we extracted the total protein and then conducted the electrophoretic separation using 10% dodecyl sulphate and sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with 50 μg of total proteins, which were then transferred to polyvinylidene fluoride (PVDF) membrane followed by blocking for 1.5 h with 5% skim milk. Primary antibodies of CCDN1 (Abcam, 1 : 10 000, #ab134175), Y567 FAK (Abcam, Eugene, Oregon, USA 1 : 50 000, #ab76120), Y861 FAK (Abcam, 1 : 10 000, #ab81293), Y397 FAK (Abcam, 1 μg/ml, #ab39967) and tubulin (Abcam, 5 μg/ml, # ab56676) were added to the membrane and stained for 24 h at 4 °C. Having washed three times using TBST, goat anti-rabbit HRP horseradish peroxidase-labelled antibodies (Abcam, 1: 2000, #ab6721) were added to incubate membranes. Electrochemiluminescence (ECL) was used for the visualization of immunoblot signals. All assays were carried out thrice.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were first placed in a 96-well plate to attach to the wells prior to experiments. At 24, 48, 72, 96 and 120 h post-transfection, 20 μl MTT (Beyotime, Shanghai, China) was supplemented into each well. Subsequently, the absorbance was determined using a microplate reader at 450 nm. Triplicate wells were measured for each treatment group.

Cell apoptosis assay

Cell apoptosis condition was detected in flow cytometry assay with propidium iodide (PI) and Annexin V double staining (BestBio, Shanghai, China). Specifically, MCF7 cells in different groups were harvested by trypsinization and resuspended at a density of 1×10^6 cells/ml. Annexin V-fluorescein isothiocyanate (FITC) and PI were applied to double staining to analyse cell apoptosis using a FACScan flow cytometer (BD Biosciences, New Jersey, USA). The above assay was carried out at least three times.

Statistical analysis

All data were analysed by means of GraphPad Prism 6.0 and presented as mean ± standard deviation ($\bar{x} \pm SD$). The differences between two different groups were compared by Student's t-test while those among multiple groups were evaluated using one-way ANOVA. Statistical significance were determined based on P value <0.05.

Results

Screening of DEGs

All DEGs were screened under the adjusted $P < 0.05$ level and $|logFC| > 1$ with DEseq2 package. Through differential gene expression analysis, 502 mRNAs showed significantly differential expressions with 338 upregulated ones and 164 downregulated ones (Figure 1a, P adj <0.05). The top 20 upregulated and downregulated mRNAs were exhibited in Figure 1b. KEGG pathway, GO biological process (BP), GO cellular component (CC) and GO molecular function (MF) chart were analysed from DAVID (Figure 1c–f). KEGG analysis showed focal adhesion pathway was significantly enriched with the $P < 0.05$ (Figure 1c). GO enrichment of BP exhibited DEGs was mostly related to microtubule-based movement and cell division (Figure 1d). GO enrichment of CC illustrated kinesin complex, nucleus and cytoplasm was most conspicuously enriched (Figure 1e). ATP binding was the most significant enriched function of MF in GO enrichment (Figure 1f). The samples performed for bioinformatics analysis including two control groups and two EGCG-treated groups, and data were obtained after 24-h incubation with the treatment of 40 μM EGCG.

GO term enrichment analysis of DEGs

GO term enrichment analysis results including GO ALL, GO BP, GO CC, GO MF varied from GO classification and expression change in DEGs. The GO analysis indicated that the overexpressed DEGs mostly enriched in nuclear nucleosome, electron transport chain, oxidative phosphorylation, *etc.* (Figure 2a). Speaking of GO BP, the upregulated DEGs mainly enriched in cell cycle arrest, cell killing, cell growth, cell adhesion, *etc.* (Figure 2b). For GO CC, the upregulated DEGs mainly enriched in snare complex, cell–cell adheren junction, DNA packaging complex, *etc.* (Figure 2c). About GO MF, the overexpressed DEGs significantly enriched in passive transmembrane transporter activity, Wnt-activated receptor activity, calcium-dependent protein binding, *etc.* (Figure 2d). DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG.

Function annotations for DEGs in EGCG-treated cells

KEGG enrichment map suggested the relationship between pathways by Cytoscape software (Figure 3a). The KEGG pathway analysis displayed that gap junction pathway, DNA replication pathway and cell cycle pathway, *etc.* were

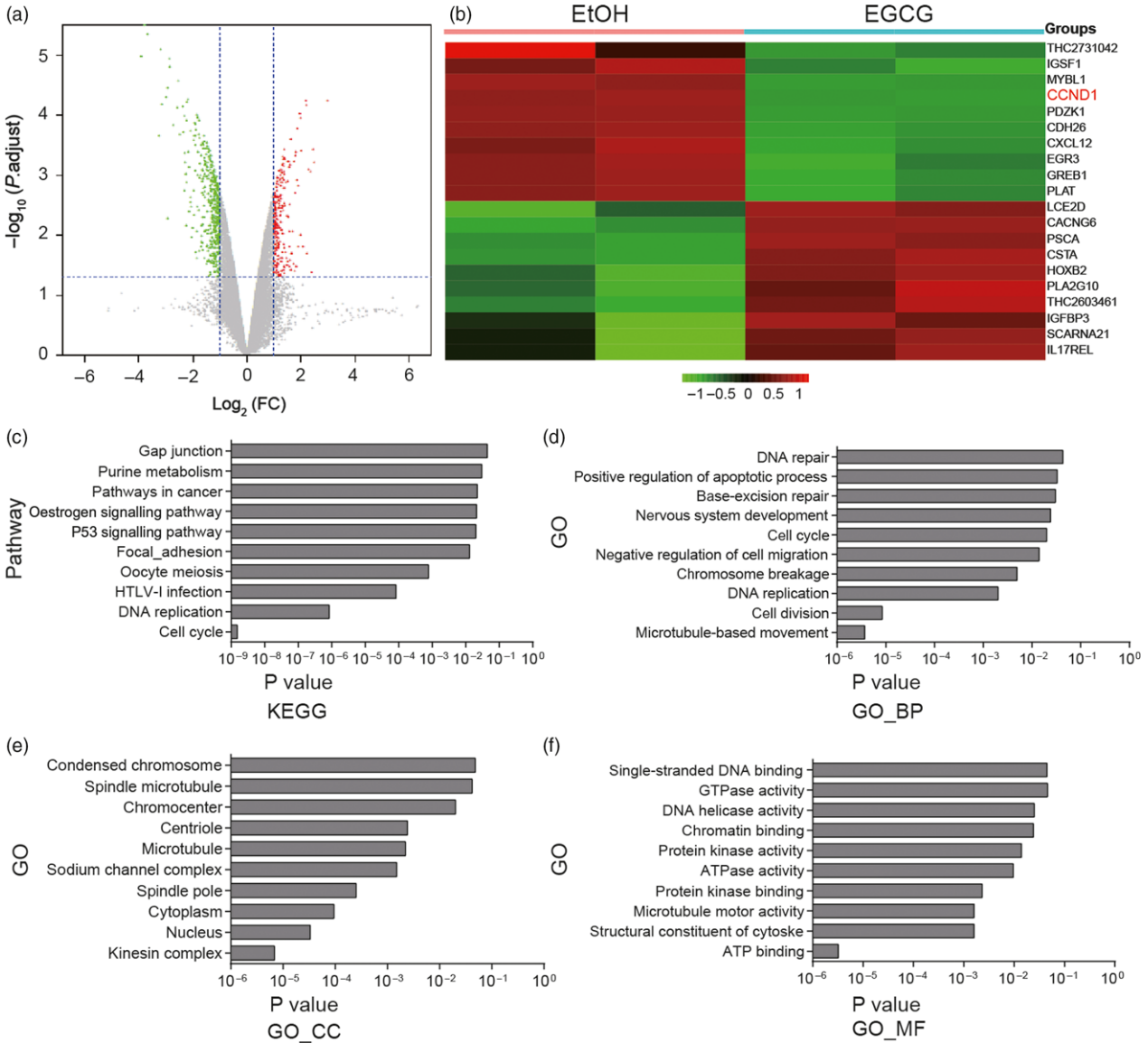

Figure 1 DEGs between two groups, KEGG pathway and GO level distribution charts. (a) The volcano plot showed the relationship between fold change and significance of mRNA expression. (b) The heatmap of 10 high-expressed mRNAs and 10 low-expressed ones in EGCG-treated MCF7 cells. The low-expressed genes from top to bottom were as follows: THC2731042, IGSF1, MYBL1, CCND1, PDZK1, CDH26, CXCL12, EGR3, GREB1 and PLAT. The high-expressed genes from top to bottom were as follows: LCE2D, CACNG6, PSCA, HOXB2, PLA2G10, THC2603461, IGFBP3, SCARNA21 and IL17REL. (c) KEGG pathway analysis of DEGs from DAVID. (d) GO biological process (BP) chart. (e) GO cellular component (CC) chart. (f) GO molecular function (MF) chart. The samples performed for bioinformatics analysis including two control groups and two EGCG-treated groups, and data were obtained after 24-h incubation with the treatment of 40 μM EGCG. [Colour figure can be viewed at wileyonlinelibrary.com]

downregulated (Figure 3b). Joyplot and dotplot of enriched KEGG pathway were analysed by ggjoy and ggplot2 packages. Following pathways were suppressed in MCF7 cells after treated with EGCG, including DNA replication pathway, focal adhesion pathway, ERBB signalling pathway, *etc.* (Figure 3c,d). Then, we calculated an enrichment score (ES) of FAK pathway that reflexed the level to which a set S is overrepresented at the top or bottom of the entire ranked list. The result showed that FAK pathway was suppressed in MCF7 cells after treatment with EGCG. The score is counted in the list, increasing an accumulated statistic when we came up against a gene in S and reducing it when we met genes not in S. The magnitude of the rise depended on the connection of the gene with the phenotype (Figure 3e). The heatmap of FAK pathway suggested that CCND1 was downregulated in this pathway

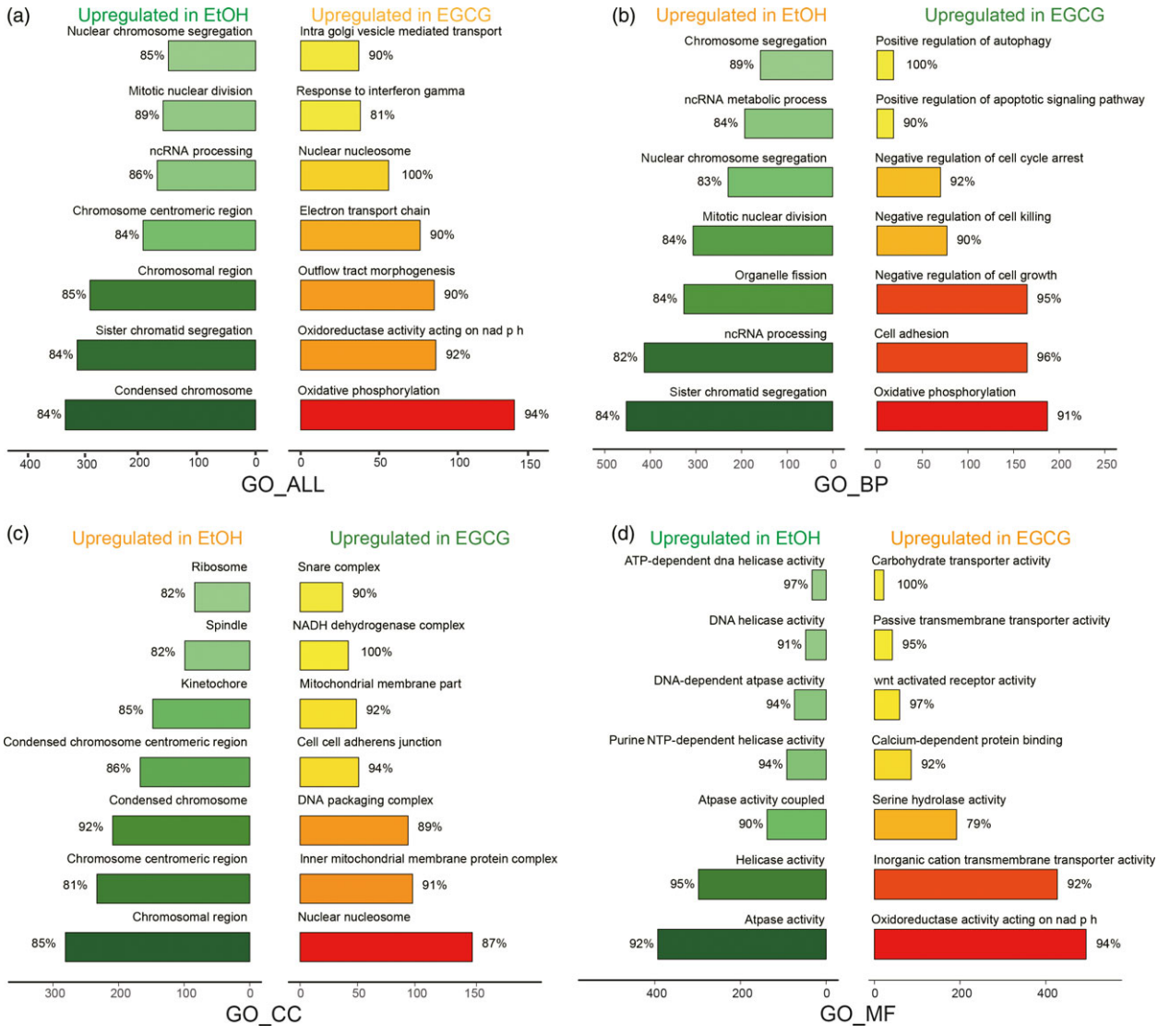

Figure 2 GO term enrichment analysis of DEGs. (a) Plot of seven most enriched pathways for GO ALL analysis. (b) Plot of seven most enriched pathways for GO BP analysis. (c) Plot of seven most enriched pathways for GO CC analysis. (d) Plot of seven most enriched pathways for GO MF analysis. DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG. [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 3f). These results indicated that EGCG could suppress FAK pathway expression. DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG.

Gene and protein networks and expression of FAK pathway

Fifty EGCG-related genes including *CCND1* were obtained from STITCH (Figure 4a). To confirm the interaction of breast cancer-related genes and KEGG pathways, protein–protein interaction (PPI) network was constructed using STRING. The network showed the enriched genes including *CCND1* in focal adhesion pathway (Figure 4b). DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG. All these bioinformatics assays were performed once.

EGCG decreased MCF7 cell proliferation and accelerated apoptosis by FAK pathway

Different EGCG concentration treatment was conducted to incubate with MCF7 cells for 24 h, and the result showed that viable cells were conspicuously reduced in 20, 40, 60 and 80 μM groups. EGCG at 40 μM concentration was ideally suited for this experiment, because it caused a modest (35%) reduction in cell viability. In this case, EGCG

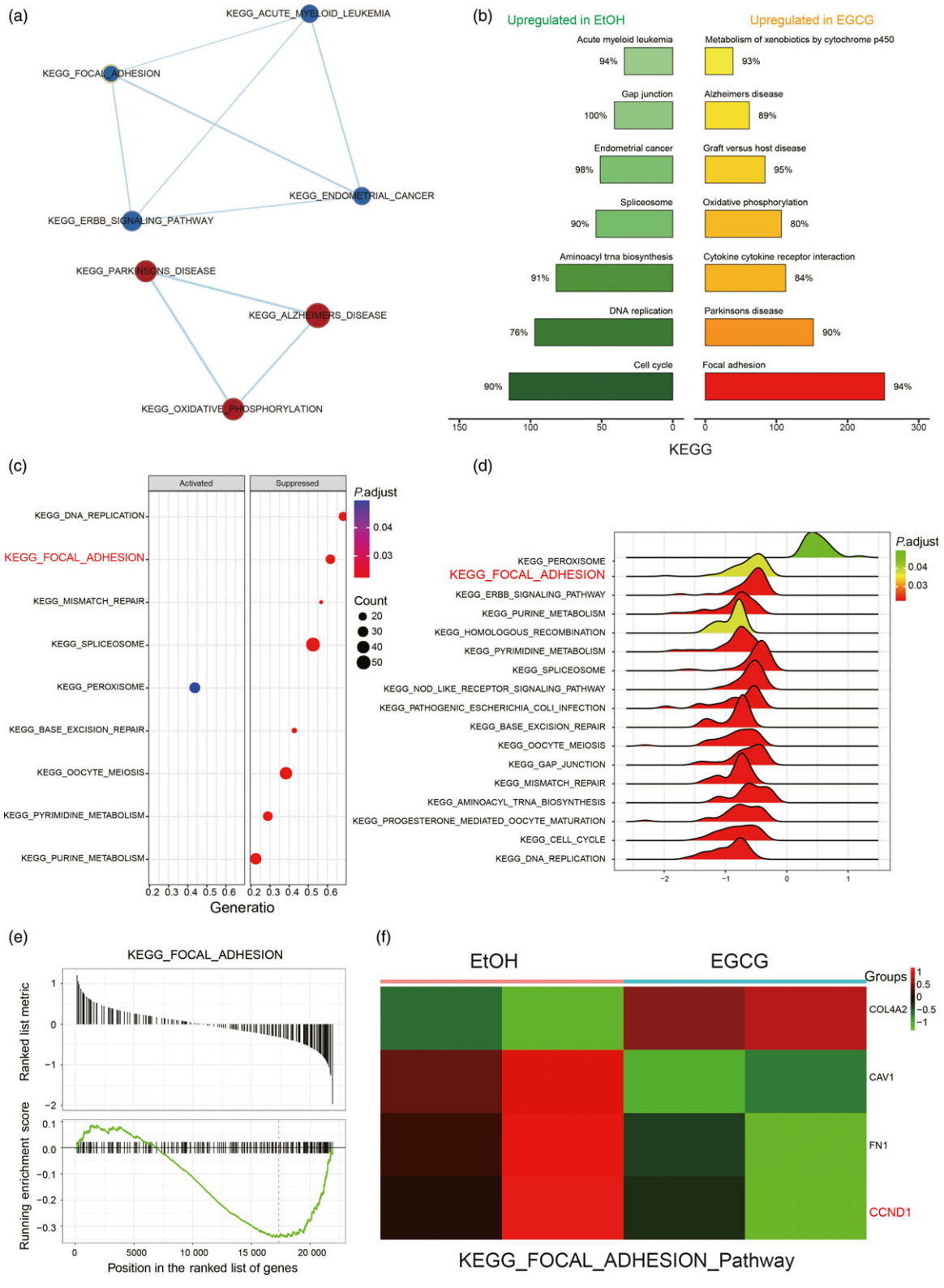

Figure 3 Function annotations for DEGs in EGCG-treated cells. (a) Enrichment map for KEGG pathway. (b) Plot of seven most significantly enriched KEGG pathways. (c, d) Joyplot and dotplot showed the distributions of a part of KEGG pathway. (e) Plot of the running enrichment score of focal adhesion pathway. (f) Gene expression of focal adhesion pathway. DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG. [Colour figure can be viewed at wileyonlinelibrary.com]

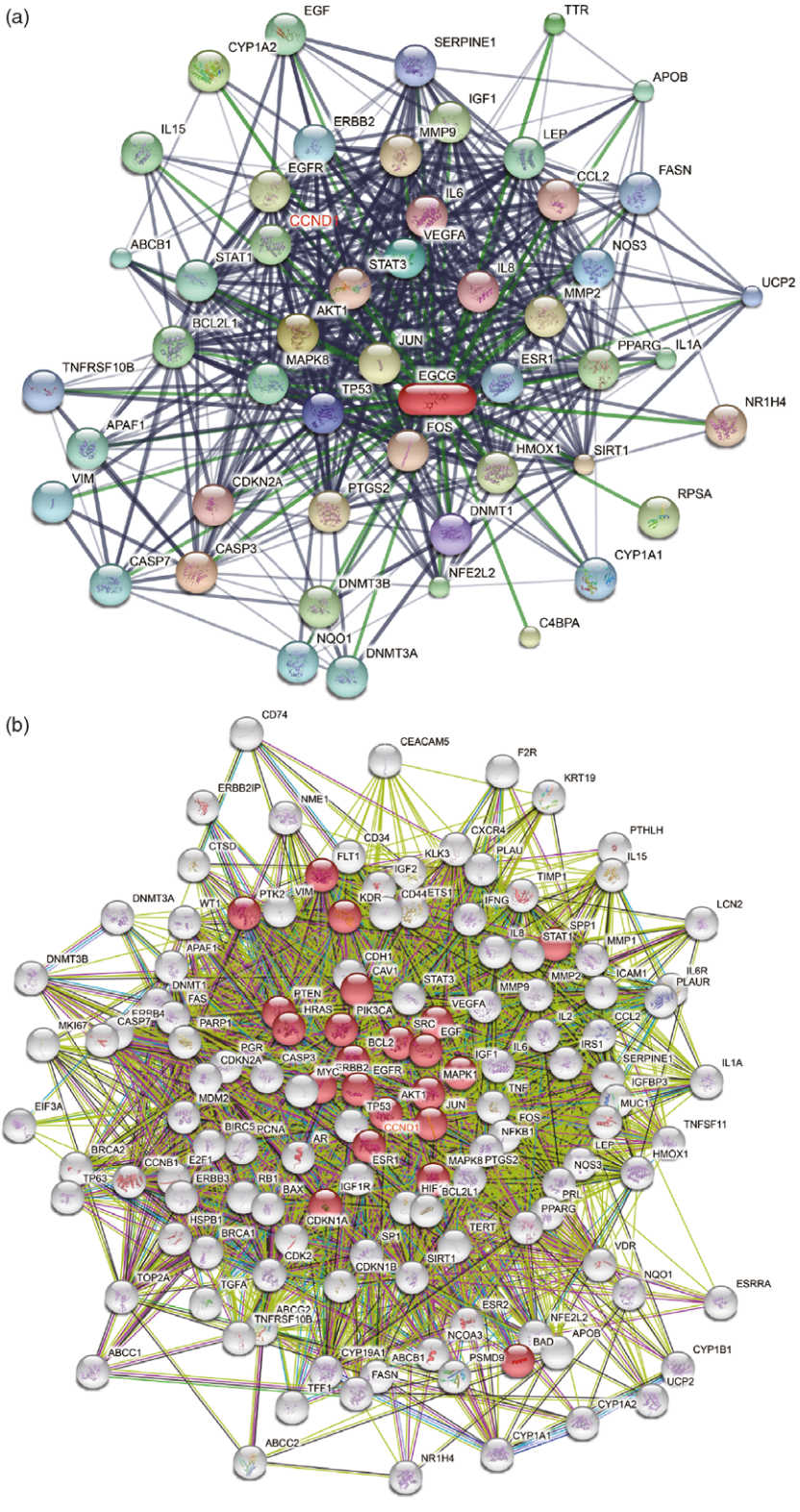

Figure 4 Gene and protein networks. (a) EGCG-related genes obtained from STITCH. (b) Protein–protein interactome constructed using STRING. DEG data were obtained after 24-h incubation with the treatment of 40 μM EGCG. All these bioinformatics assays were performed once. [Colour figure can be viewed at wileyonlinelibrary.com]

regulation of gene expression could be safely interpreted as primary rather than as a side effect of cytotoxicity observed at high concentrations (Figure 5a, $P < 0.05$, $P < 0.01$). QRT-PCR results detected that CCND1 was significantly downregulated in MCF7 cells after treated with EGCG (Figure 5b, $P < 0.05$). MTT assay indicated a lower curve in EGCG-treated group in comparison with control group (Figure 5c, $P < 0.05$). In order to detect whether EGCG directly interacted or inhibit CCND1 protein expression, Western blot assay was performed. The result showed that CCND1 protein expression level was significantly reduced by EGCG (Figure 5d, $P < 0.01$). To study whether EGCG also suppressed expression of protein in FAK pathway, the expression of phosphorylation of FAK at Y576, Y861 and the autophosphorylation site Y397 was examined through Western blot, of which the results displayed that phosphorylated FAK expression was significantly downregulated in EGCG-treated group (Figure 5e, $P < 0.05$). The apoptosis rate of MCF7 cells was increased after treated with EGCG (Figure 5f, $P < 0.05$). These results implied that EGCG could decrease MCF7 cell proliferation and accelerated apoptosis by FAK pathway. The experiments were

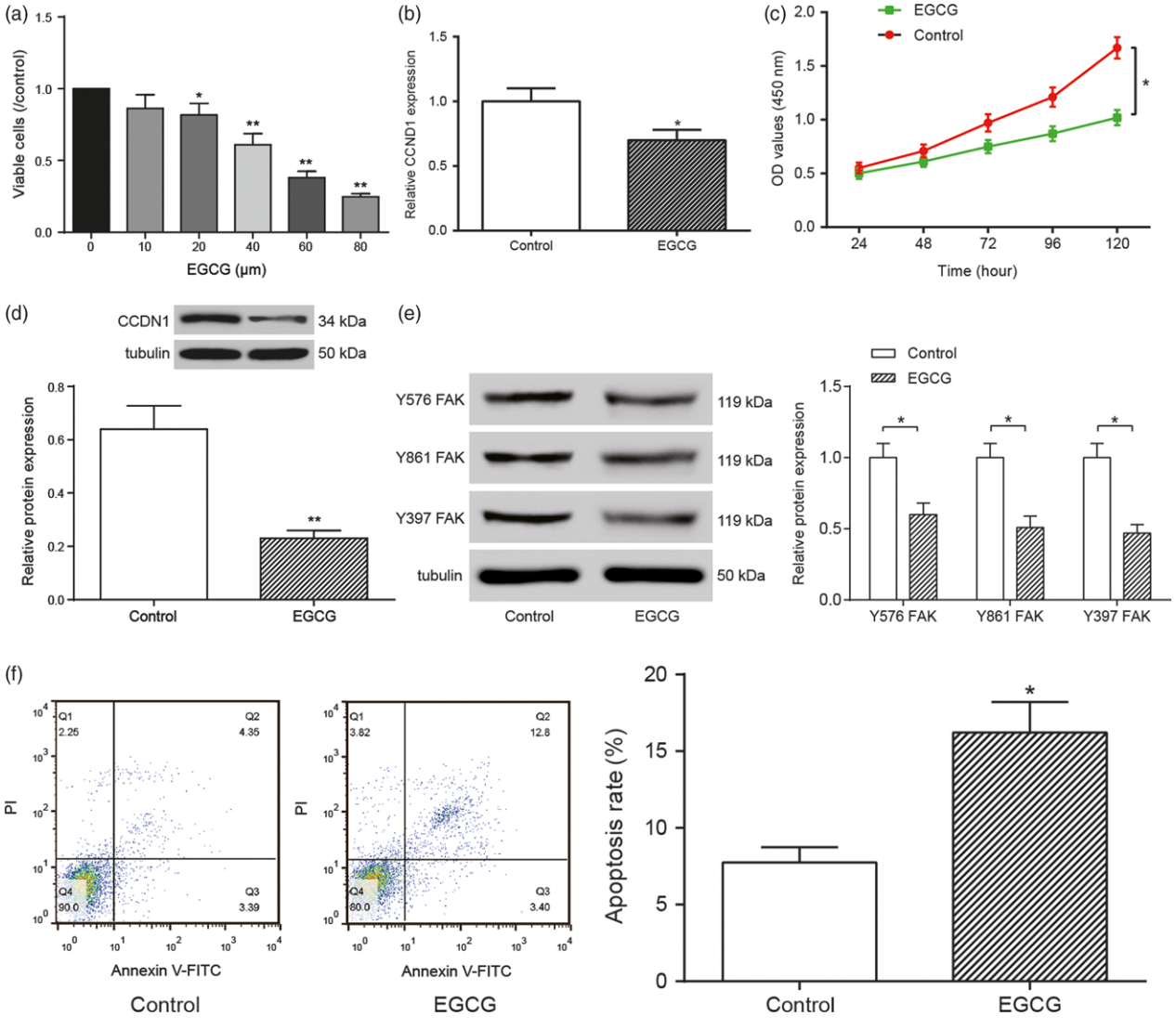

Figure 5 EGCG decreased MCF7 cell proliferation and accelerated apoptosis by FAK pathway. (a) Cell viabilities detecting under different concentration EGCG treatment. (b) The *CCND1* expression level was downregulated in EGCG-treated group compared with control group. (c) MTT assay demonstrated that EGCG inhibited MCF7 cell proliferation. (d) EGCG inhibited CCND1 protein expression level of MCF7 cells. (e) EGCG suppressed expressions of phosphorylation of FAK at Y576, Y861 and the autophosphorylation site Y397 by Western blot. (f) EGCG accelerated MCF7 cell apoptosis. *$P < 0.05$, **$P < 0.01$, compared with control group. The experiments were performed after 24-h incubation with the treatment of 40 μM EGCG, and every experiment was performed for three times at least. [Colour figure can be viewed at wileyonlinelibrary.com]

performed after 24-h incubation with the treatment of 40 μM EGCG, and every experiment was performed for three times at least.

Overexpression of *CCND1* in EGCG-treated MCF7 cells reversed the effect of EGCG on MCF7 cells

After transfected with pEGFP-N1-*CCND1*, the expression of *CCND1* was significantly upregulated in EGCG-treated MCF7 cells (Figure 6a, $P < 0.05$). MTT assay showed that the proliferation of EGCG-treated MCF7 cells was markedly increased after upregulating *CCND1* (Figure 6b, $P < 0.05$). Simultaneously, the apoptosis of pEGFP-N1-*CCND1* group was considerably lower in contrast to MOCK and NC group (Figure 6c,d, $P < 0.05$). These results indicated that overexpression of *CCND1* could reverse the effect of EGCG on MCF7 cells. The experiments were performed after 24-h incubation with the treatment of 40 μM EGCG, and every experiment was performed for three times at least.

Discussion

Considerable evidence suggested that EGCG could regulate related genes to exert influence on progression of several types of cancer. For instance, Feng *et al.*[34] verified that epigallocatechin gallate restrained cell growth and promoted apoptosis in bladder cancer through modulating TFPI-2. Meng *et al.*[35] found that EGCG inhibited cell viability and induced apoptosis in oesophageal cancer by reactivating p16. Gu *et al.*[20] disclosed that EGCG restrained breast tumour angiogenesis and growth through repressing activation of HIF-1α and NFκB, and VEGF expression. In this research, we identified key signalling pathways and related differential gene *CCND1* based on bioinformatics tool and pathway enrichment and network analyses. Through in-vitro experiments, we confirmed that CCND1

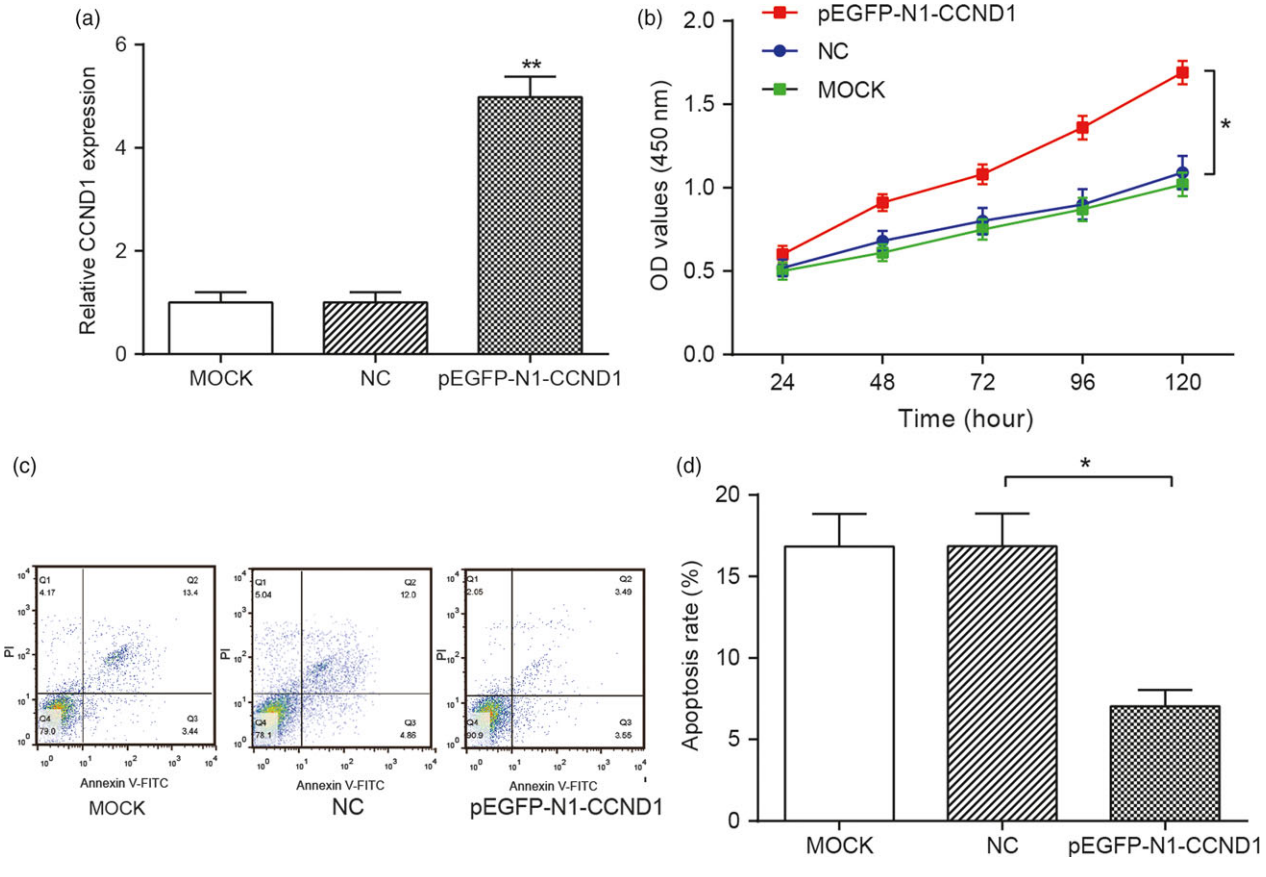

Figure 6 Overexpression of *CCND1* in EGCG-treated MCF7 cells reversed the effect of EGCG on MCF7 cells. (a) The *CCND1* expression level was considerably upregulated after transfection with *CCND1* pEGFP1-N1-CCND1. (b) MTT assay showed that overexpression of *CCND1* promoted EGCG-treated MCF7 cell proliferation. (c, d) Overexpression of *CCND1* decreased cell apoptosis. *$P < 0.05$, **$P < 0.01$, compared with control group. The experiments were performed after 24-h incubation with the treatment of 40 μM EGCG, and every experiment was performed for three times at least. [Colour figure can be viewed at wileyonlinelibrary.com]

overexpression had an oncogenic effect on breast cancer cells, while EGCG inhibited breast cancer cell viability and accelerated apoptosis by modulating CCND1, which further verified the anticancer function of EGCG in breast cancer. Moreover, in our study, cell activity was detected after different concentration treatment of EGCG and the treatment group of 40 μM was selected for further experiments. According to other EGCG-related researches, the EGCG concentration of 40–60 μM was frequently used. Li *et al.*[36] explored EGCG inducing on lung cancer A549 cell apoptosis in 40 μM dosage, and Amicis *et al.*[37] applied 40 μM dosage to research the mechanism of EGCG affecting on human breast cancer cells, etc.

In addition, accumulating studies have reported that many dietary cancer chemopreventive agents including polyphenols interfered with the carcinogenic process by modulating one or more cell signalling pathways.[38] EGCG and other compounds extracted from green tea have been shown to exert a certain influence on cell signalling pathways. For instance, Senggunprai *et al.*[39] disclosed that EGCG exhibited chemopreventive effects on cholangiocarcinoma cells by repressing JAK/STAT signalling pathway. Albrecht *et al.*[40] proved that epigallocatechin-3-gallate inhibited PC-3 prostate cancer cell proliferation via a PI3-K-dependent signalling pathway. Furthermore, Rathore *et al.*[6] revealed that green tea catechin including EGCG could intervene reactive oxygen species-mediated ERK pathway activation and impede breast cancer carcinogenesis. Huang *et al.*[22] also unravelled that EGCG suppressed the proliferation of human MCF7 breast cancer cells and facilitated apoptosis via P53/Bcl-2 signalling pathway. Herein, we identified the mechanism of FAK signalling pathway involving in EGCG-mediated cell propagation and apoptosis and verified that EGCG could impede MCF7 cell proliferation and accelerated apoptosis via FAK pathway *in vitro*.

Our pathway analysis indicated that ECGC could affect development and progression of breast cancer through the focal adhesion kinase signalling pathway. Focal adhesion kinase (FAK), a member of the FAK subfamily of protein tyrosine kinases, encodes a cytoplasmic protein tyrosine kinase concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents.[41] *FAK* gene activation may exert important effects on cell growth and intracellular signal transduction pathways perturbed in response to certain neural peptides or to cell interactions with the extracellular matrix. Accumulating researches demonstrated that inhibition of FAK signalling contributed to suppressing cellular events in various types of cancers including melanomas,[42] ovarian cancer,[43] non-small-cell lung cancer[44] and breast cancer.[45] Our network analysis implicated several pathways including FAK signalling pathway through which EGCG might prevent breast cancer progression, involving cell proliferation and apoptosis. These results are consistent with several studies *in vitro* and *in vivo* suggesting that EGCG exerts anticarcinogenic activity in breast cancer.[46]

Clinical studies suggested the role of EGCG, which suppressed tumour growth by blocking receptors in affected cells. Another possible mechanism suggested that EGCG may promote direct binding to certain carcinogenic substances.[47] These results were consistent with the initial hypothesis in our study that EGCG affects the development of breast cancer by acting on CCND1. A prospective cohort study with over 8000 individuals showed that the daily consumption of green tea led to delayed cancer onset, and a follow-up study of breast cancer patients revealed that stages I/II breast cancer patients experienced a lower recurrence rate.[48,49] From a clinical perspective, there were many studies that supported the anticancer effect of EGCG and cancer incidence would be reduced by daily consumption of EGCG and other catechins, which were also consistent with our findings.

Nonetheless, there were still some limitations worthy to be mentioned in our experiments. First, we examined the mechanism of FAK pathway in the EGCG-modulated cell proliferation and apoptosis through in-vitro assay only. Second, our bioinformatics analysis of microarray profiling did not implicate CCND1 upstream miRNAs and lncRNAs. Therefore, we would conduct an intensive study on this aspect in the future research.

In conclusion, we delved into the mechanism of FAK signalling pathway in proliferation and apoptosis of breast cancer cells modulated by green tea polyphenol EGCG based on bioinformatics analysis of microarray profiling and identified the cellular signalling mechanism responsible for the anticancer effects of EGCG in MCF7 cells. In the present research, we integrated data from GEO data portal and identified 502 DEGs between EGCG-treat group and control group. We found that DEGs mostly enriched in gap junction, DNA replication, cell cycle, oxidative phosphorylation and focal adhesion using GO and KEGG pathway enrichment analyses. After treatment with EGCG, some pathways including FAK signalling pathway were significantly suppressed. Through network analysis, we identified a set of significantly differential hub genes including *CCND1* enriched in FAK signalling pathway and CCND1 was remarkably downregulated in EGCG-treated group, suggesting that EGCG could repress CCND1 expression via FAK signalling pathway. Ultimately, we substantiated that EGCG might restrain breast cancer cell propagation and promote apoptosis by inhibiting FAK signalling pathway in-vitro experiment. The above findings further validated the potential therapeutic value of EGCG in breast cancer and provided an effective adjuvant therapy in the treatment of breast cancer.

Conclusions

A total of 502 mRNAs were identified as DEGs based on microarray analysis. GO term enrichment analysis suggested that upregulated DEGs mainly enriched in nuclear nucleosome, cell adhesion, DNA packaging complex, Wnt-activated receptor activity, *etc*., while the downregulated DEGs significantly enriched in ncRNA processing, mitotic nuclear division, DNA helicase activity, *etc*. KEGG pathway analysis indicated that DEGs mostly enriched in gap junction, DNA replication, cell cycle, oxidative phosphorylation, focal adhesion, *etc*. QRT-PCR results indicated CCND1 expression was significantly downregulated in EGCG groups. Western blot assay suggested that EGCG suppressed FAK signalling pathway. Furthermore, EGCG could inhibit breast cancer cell proliferation and promote apoptosis by modulating CCND1. EGCG might exert influence on breast cancer progression through inhibiting focal adhesion kinase (FAK) signalling pathway based on bioinformatics analysis.

Declarations

Conflict of interest

The Authors declare that they have no conflict of interests with the contents of this article.

Acknowledgements

This work was supported by Science and Technology Development Plan Project of Jilin Province. (Grant number 20150204082SF).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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