Multiple effects of ellagic acid on human colorectal carcinoma cells identified by gene expression profile analysis

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Received August 17, 2016; Accepted November 25, 2016

DOI: 10.3892/ijo.2017.3843

Abstract. Colorectal carcinoma (CRC) is the third most commonly diagnosed cancer in the world. Phytochemicals have become a research hotspot in recent years as cancer prevention and treatment agents due to their low toxicity and limited side-effects. Ellagic acid (EA), a natural phenolic constituent, displays various biological activities, including anticancer effects. However, the detailed anticancer mechanisms of EA remain unclear. In the present study, we found that EA inhibited the growth of HCT-116 colon cancer cells. Moreover, we identified differentially expressed genes (DEGs) by microarray profiling of HCT-116 cells treated with EA. A total of 857 DEGs (363 upregulated and 494 downregulated) were identified with a >1.5-fold change in expression after treatment with EA for 72 h. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that a large number of cellular functions were modified by EA including proliferation, apoptosis, cell cycle and angiogenesis. Interaction network analysis using DEGs provided details of their interactions and predicted the key target pathways of EA. To verify the result of cDNA microarray, 10 selected DEGs related to proliferation, apoptosis or cell cycle were further confirmed by real-time RT-PCR. Based on microarray data, we identified several crucial functions of EA. These results provide important new data for EA in anti-CRC research.

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Key words: ellagic acid, colorectal carcinoma, microarray profiling, differentially expressed genes, Gene Ontology analysis, Kyoto Encyclopedia of Genes and Genomes pathway analysis, multi-target

Introduction

Colorectal carcinoma (CRC) is the third most common cancer and the second leading cause of tumor related deaths in the world (1). Given the global distribution of morbidity from CRC, it is apparent that the environmental factors play a significant role in its etiology. The connection between the diet and the incidence of CRC is now well-established (2). Although surgery, radiotherapy, chemotherapy and targeted therapies for CRC are improving, the existing treatment methods cannot completely control the high incidence or low survival rates of CRC. Chemoprevention has emerged recently as one of the most practical and effective tools to reduce the risk of cancer (3). Bioactive substances from plants are a source for novel antitumor drugs, and polyphenolic compounds, in particular, have been the focus of increasing interest due to their strong anticancer activity (4,5).

Ellegic acid (EA; 2,3,7,8-tetrahydroxy-chromeno [5,4, 3-cde] chromene-5,10-dione; International Union of Pure and Applied Chemistry) is a polyphenolic compound abundant in woody plants, berries, grapes and nuts (6). EA has been found to exert both preventive and therapeutic effects against numerous human types of cancer, including colon, skin, prostate, breast and esophageal cancer (7-10). A number of studies have investigated the mechanisms of EA in the inhibition of carcinogenesis. Recent research has demonstrated that it suppresses cancer cell proliferation and migration by downregulation of VEGF-induced angiogenesis, VEGF-2 tyrosine kinase activity, and downstream MAPK and PI3K/Akt signaling pathways (10). EA also inhibits the invasive potential of tumors through its effects on the activity of proteases, such as collagenase/gelatinase and collagenase IV (11). In addition, EA can reduce the cancer cell viability by increasing the caspase-3 activity, downregulating Bcl-2 and decreasing the activity of telomerase (12). Although these studies focus on the mechanisms of EA in various signaling pathways, they failed to comprehensively encompass all of its biological activities. Moreover, the molecular effects of EA in inhibition of human CRC cells remain to be thoroughly elucidated.

In the present study, we used a high-throughput GeneChip containing >20,000 known genes to identify multiple targets affected by EA in human colon adenocarcinoma HCT-116 cells. This cDNA microarray method detects changes in gene

expression profiles, providing evidence for the effects of anti-cancer agents on cancer cells (13). The GeneChip results were further confirmed by the real-time RT-PCR. Multiple different functions of EA were revealed in human CRC cells, providing vital data that will be of significant value to researchers.

Materials and methods

EA and cell lines. EA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A stock solution of EA was prepared in dimethyl sulfoxide (DMSO) and filter sterilized before use. The human CRC cell line, HCT-116, was purchased from the Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). HCT-116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin solution in an atmosphere of 95% air and 5% CO_2 in a 37˚C humidified incubator.

Cell proliferation assay. HCT-116 cells were seeded in 96-well plates at a density designed to reach ~80% confluence. Cells were allowed to adhere and 24 h later were treated with EA at 0, 25, 50, 75, 100 and 125 μM. After 24, 48 or 72 h of treatment, 10 μl of MTT was added to 100 μl culture medium per well. After 4 h of incubation at 37˚C, the medium was removed and 150 μl DMSO was added. The absorbance was measured at a wavelength of 490 nm in a plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated according to: OD sample/OD control x 100%. The assay was performed in triplicate.

RNA extraction. After 72 h of EA treatment, total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity and integrity of the ribosomal RNA was checked.

Transcriptome microarray assay. Affymetrix Human Transcriptome Array 2.0 arrays (HTA 2.0) were hybridized according to the Affymetrix recommendations, using the Ambion WT protocol and Affymetrix Labeling and Hybridization kits. GeneChips were scanned using Affymetrix® GeneChip Command Console (AGCC). Data were manipulated using the Expression Console software (Affymetrix) at the Institut Curie Microarray Core Facility.

Differential gene expression analysis. A random variance model t-test was used to filter differentially expressed genes (DEGs) between the the control and the experimental groups. We selected DEGs according to a P-value threshold corrected using the false discovery rate (FDR) method, and a corrected $P<0.05$ was considered statistically significant (14-16).

Gene Ontology (GO) analysis. To analyze the main functions of the DEGs identified by microarray analysis, we used the Gene Ontology (the key functional classification from NCBI), which organizes genes into hierarchical categories and reveals regulatory networks based on the molecular functions and biological processes (17,18). Due to the hierarchical relationships between the GO terms, the number of genes in each term varies widely and can be between one and several hundred genes. For a given marginal frequency (i.e. a constant total number of genes in a GO term), the probability of a gene being affected by a treatment obeys a hypergeometric distribution and P-values can be calculated by Fisher's exact test. Essentially, the P-value of a two-tailed Fisher's exact test solves the cumulative hypergeometric distribution values.

Specifically, two-sided Fisher's exact and χ^2 tests were used to analyze GO categories, and the FDR was used to correct P-values (19). Scores to assess the enrichment of GOs were calculated using the following formula:

$$\mathbf{Re} = \left(\frac{n_f}{n}\right) / \left(\frac{N_f}{N}\right)$$

where 'n_f' is the number of DEGs in a GO, 'n' is the number of genes in a GO, 'N_f' is the total number of DEGs, and 'N' is the total number of genes in the annotation system (20).

Pathway analysis. Pathway analysis is used to determine the significant pathways in which DEGs participate. Since entire biochemical processes, including metabolism, signal transduction and the cell cycle, are described as 'pathways', a single pathway usually contains hundreds of genes. Fisher's exact and χ^2 tests were used to detect regulatory pathways differing significantly according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta and Reactome, and significance thresholds were defined by FDR-corrected P-values (21-23).

Interaction networks of DEGs. Gene network analysis using the KEGG database is used to construct systems of interactions and overcome the limitations of determining interactions among genes in a single pathway. Genes with discordant records of upregulation or downregulation of mRNA expression in the database were excluded from the analysis and were recorded as having a negative association by default, which was not taken into account in multi-group differentiation analysis. The intersection was taken as the result. Therefore, gene signaling network analysis was able to determine the upstream and downstream molecules for proteins throughout the KEGG pathway database.

An interaction network was constructed based on the DEG data. Network maps were constructed using Java, which allows users to build and analyze molecular networks. Analysis was based on the KEGG interaction database. Networks were stored and presented as graphs, where nodes are mainly genes (proteins and complexes) and edges represent types of relationships between nodes, such as activation or phosphorylation.

The degree is defined as the number of links of one node with all other nodes. For a gene in a network, the number of source genes connecting to a gene is called its indegree, while the number of target genes connected to by a gene is its outdegree. The properties of genes are described by measures of betweenness centrality, which reflects the importance of a node in modulation of other nodes (24-28).

Real-time reverse transcription-PCR analysis. Changes in the expression of ten selected genes responding to EA were further assessed by quantitative RT-PCR. A total of 2 μg of RNA from independent experiments was used to perform reverse

Table I. The primers used for real-time RT-PCR analysis.

Gene	Primer sequence
β-actin	CTCACCATGGATGATGATATCGC
	AGGAATCCTTCTGACCCATGC
IL8	CACCGGAAGGAACCATCTCA
	TGGCAAAACTGCACCTTCACA
JUN	CCAACTCATGCTAACGCAGC
	CTCTCCGTCGCAACTTGTCA
CCNB1	TGGTGAATGGACACCAACTCT
	TAGCATGCTTCGATGTGGCA
IRS1	ACATCACAGCAGAATGAAGACCT
	TGGATGCATCGTACCATCTACTG
PLK1	CAAGTACGGCCTTGGGTATCA
	GTGCCGTCACGCTCTATGTA
CDC20	ATTCCCAGGTGTGCTCCATC
	GCCATGGTTGGGTACTTCCA
SMC3	CAGACAACCGGTTACCAATCG
	AGCGCTTTCAAGGAGGTTCA
BCL-2	AGATTGATGGGATCGTTGCCT
	AGTCTACTTCCTCTGTGATGTTGT
BAD	TCCTTTAAGAAGGGACTTCCTCG
	CCAAGTTCCGATCCCACCAG
C-MYC	CCTACCCTCTCAACGACAGC
	TTCCTCCTCAGAGTCGCTGC

transcription using PrimeScript™ RT reagent kit (Takara Bio, Tokyo, Japan). Real-time quantitative PCR of transcribed cDNA was performed with SYBR Premix Ex Taq™ II (Takara Bio). Primers were designed using Primer 5 software and synthesized by Comate Bioscience, Co., Ltd. (Changchun, China). The primers are listed in Table I. Real-time RT-PCR reactions were then performed in a total of 25 μl of reaction mixture using the ABI Prism 7500HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the comparative Ct method, and the expression levels of target genes were normalized to the levels of β-actin expression in each sample.

Statistical analysis. All data were presented as the mean ± SD of six independent experiments. Two-tailed Student's t-test and one-way analysis of variance (ANOVA) were used to analyze significant differences. P<0.05 was considered to indicate a statistically significant result.

Results

The effects of EA on HCT-116 cell proliferation. MTT assays were used to assess the anti-proliferative effects of EA and to select the appropriate concentration of EA and treatment duration for the microarray assay. As shown in Fig. 1, HCT-116 cells were incubated with different concentrations of EA for 24, 48 or 72 h. EA exhibited anti-proliferative effects, which were both time- and dose-dependent.

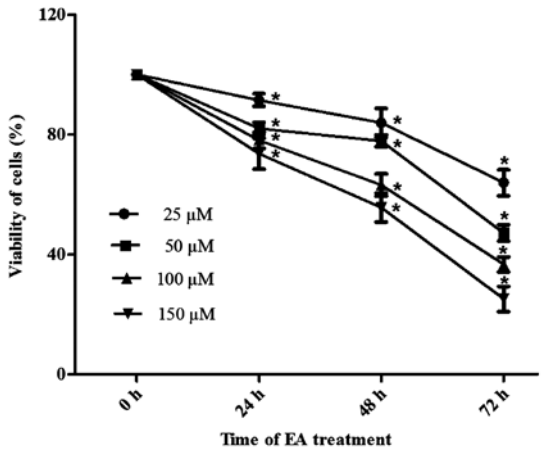

Figure 1. Effect of EA on the growth of HCT-116 cells. HCT-116 cells were exposed to increasing dosages of EA for 24, 48 or 72 h. Cell viability is expressed as a percentage of the control group (DMSO). Data are presented as means ± SD, n=6 per group. *P<0.05 compared to the vehicle control.

Screening for differentially expressed genes. After treatment of HCT-116 cells with 100 μM EA for 72 h, microarray analysis indicated that a total of 857 genes had expression levels changed by ≥1.5-fold (494 upregulated and 363 downregulated) (Fig. 2; P<0.05). The top 10 DEGs are presented in Table II.

GO analysis. Comprehensive GO analysis of DEGs was performed to determine the main functions of EA in cancer inhibition. Thirty-eight GO terms significantly associated with differential gene expression after EA treatment (Fig. 3; P<0.05) were classified into key functional categories. The main GO categories identified included regulation of the apoptotic process, cell division, signal transduction, negative regulation of cell proliferation, gene expression, transcription and cell cycle arrest. Larger enrichment values indicate that the functions were affected more strongly by EA treatment.

Pathway analysis of DEGs. KEGG pathway analysis was performed to further investigate the key pathways associated with DEGs. Significant pathway categories (P<0.05) for the 857 DEGs linked to EA are presented in Fig. 4. Larger -Lg (P-values) indicate that the function was more strongly regulated in response to treatment with EA. Treatment with EA clearly affected 24 significant pathways, including the p53 signaling pathway, metabolic pathways, the PI3K-Akt signaling pathway, the MAPK signaling pathway, the TGF-β signaling pathway and the cell cycle and transcriptional misregulation in cancer.

Interaction networks of DEG. Interaction-relationship networks of DEGs were constructed and are shown in Figs. 5 and 6. DEGs of interest were closely connected, and the majority of them were located in the center of the network. Betweenness centrality indicated the intermediary ability of each gene, and a larger value indicates a greater ability to

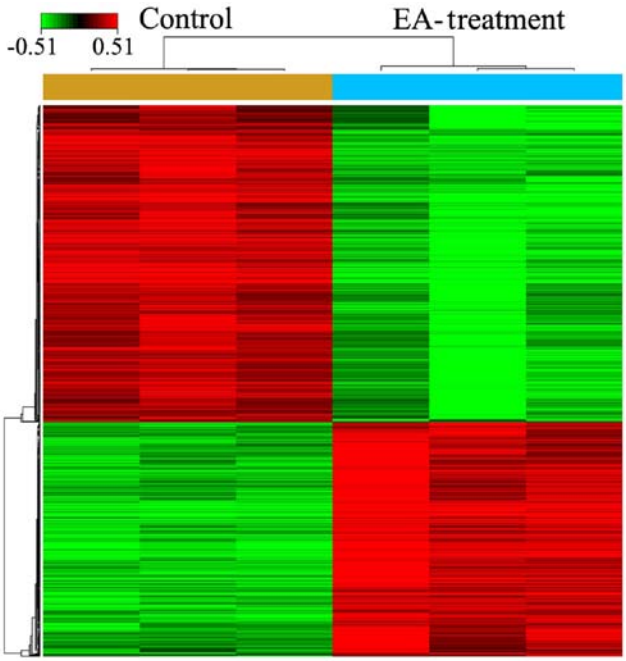

Figure 2. Heat map of DEGs with fold change expression >1.5. HCT-116 cells were treated with 100 μM EA for 72 h (n=3) or treated with vehicle control (n=3). Rows represent genes and columns represent samples. Red blocks represent high and green blocks low expression relative to comparison cells.

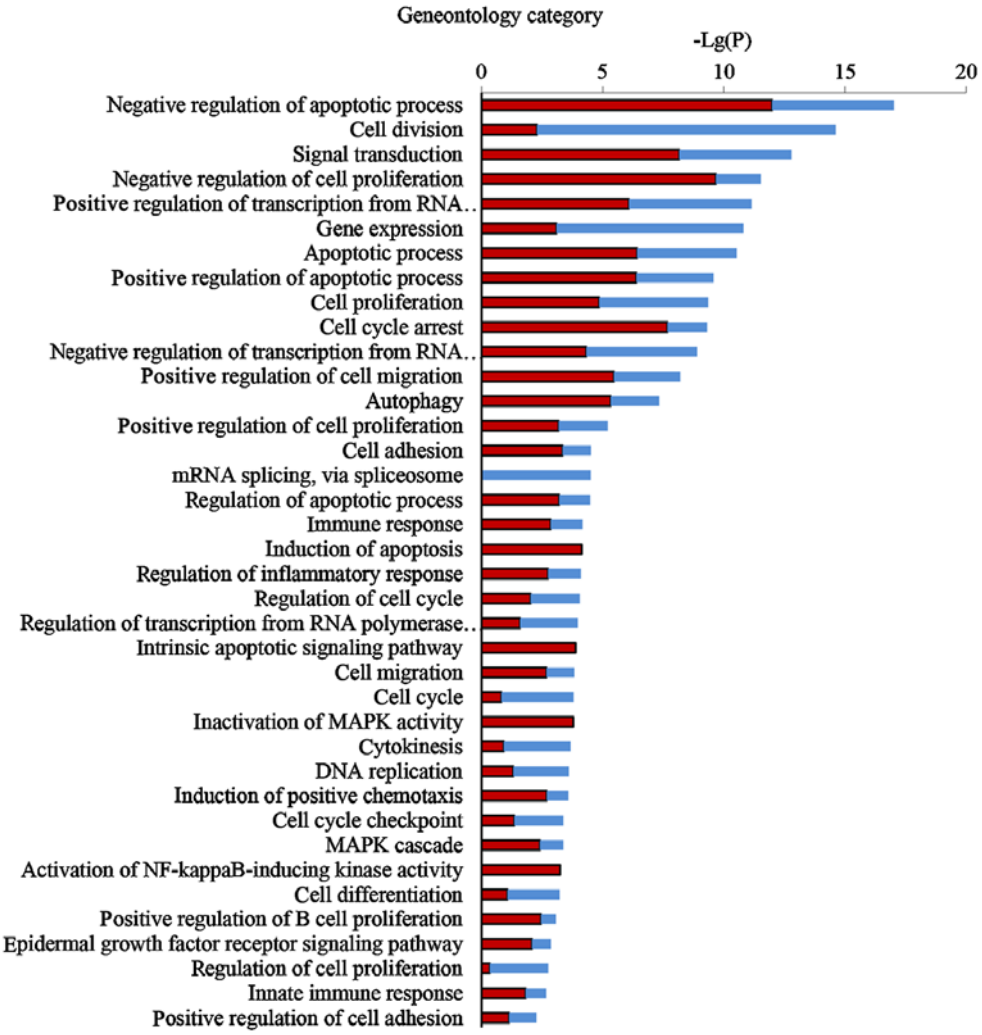

Figure 3. Enrichment of GO categories. Blue columns indicate the percentage of upregulated genes and red columns indicate the percentage of downregulated genes in each category.

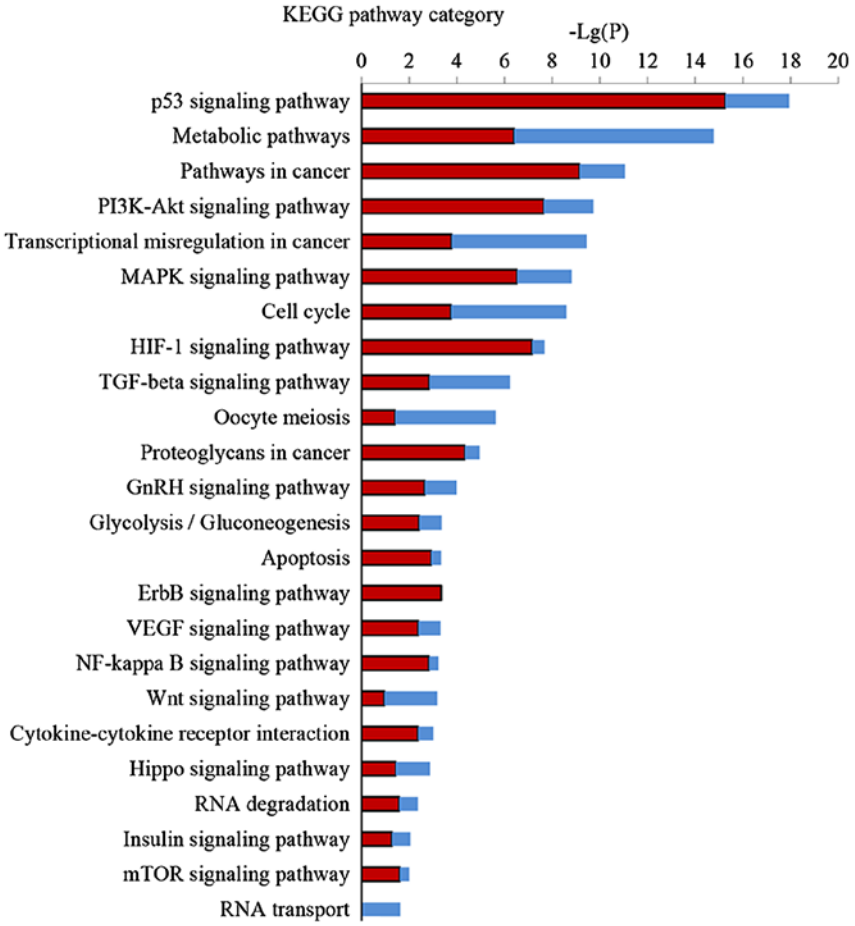

Figure 4. KEGG pathway analysis of HCT-116 cells after EA treatment. Blue columns indicate the percentage of upregulated genes and red columns indicate the percentage of downregulated genes in each pathway.

Table II. Top 10 regulated genes in EA-treatment cells compared with the control cells in the HCT-116 cells.

Rank	Gene symbol	Gene feature	Fold change	P-value
1	CPA4	Upregulated	7.393308	5.10E-05
2	ABCC2	Downregulated	-6.93316	5.60E-05
3	CENPE	Downregulated	-5.35232	6.10E-05
4	CENPF	Downregulated	-4.48105	8.00E-05
5	BHLHE40	Upregulated	4.231785	7.00E-05
6	HIST1H2BM	Downregulated	-4.22724	9.50E-05
7	KRTAP2-3	Upregulated	4.124822	0.000148
8	ID3	Downregulated	-3.97283	0.000163
9	GDF15	Upregulated	3.684665	0.000265
10	HIST1H2AB	Downregulated	-3.65796	0.00025

regulate genes. Fig. 6 shows the interaction network of DEGs related to proliferation, apoptosis, angiogenesis and cell cycle. These DEGs include a number of important functional genes such as PRKACB, IL8, JUN, CDC20 and CCNB1.

Real-time reverse transcription-PCR analysis. The next step was to confirm the changes of genes in microarray analysis by the real-time RT-PCR analysis. We selected 10 genes (3 upregulated, 6 downregulated and 1 no change by microarray data) which were related to proliferation, apoptosis or cell cycle. Nine out of the ten gene expression levels were verified by the real-time RT-PCR. This represents a success rate of 90% in microarray analysis. However, when the relative ratio of change for genes in microarray exceeded 2, the success rate became 100% (7 out of 7). The results are shown in Table III.

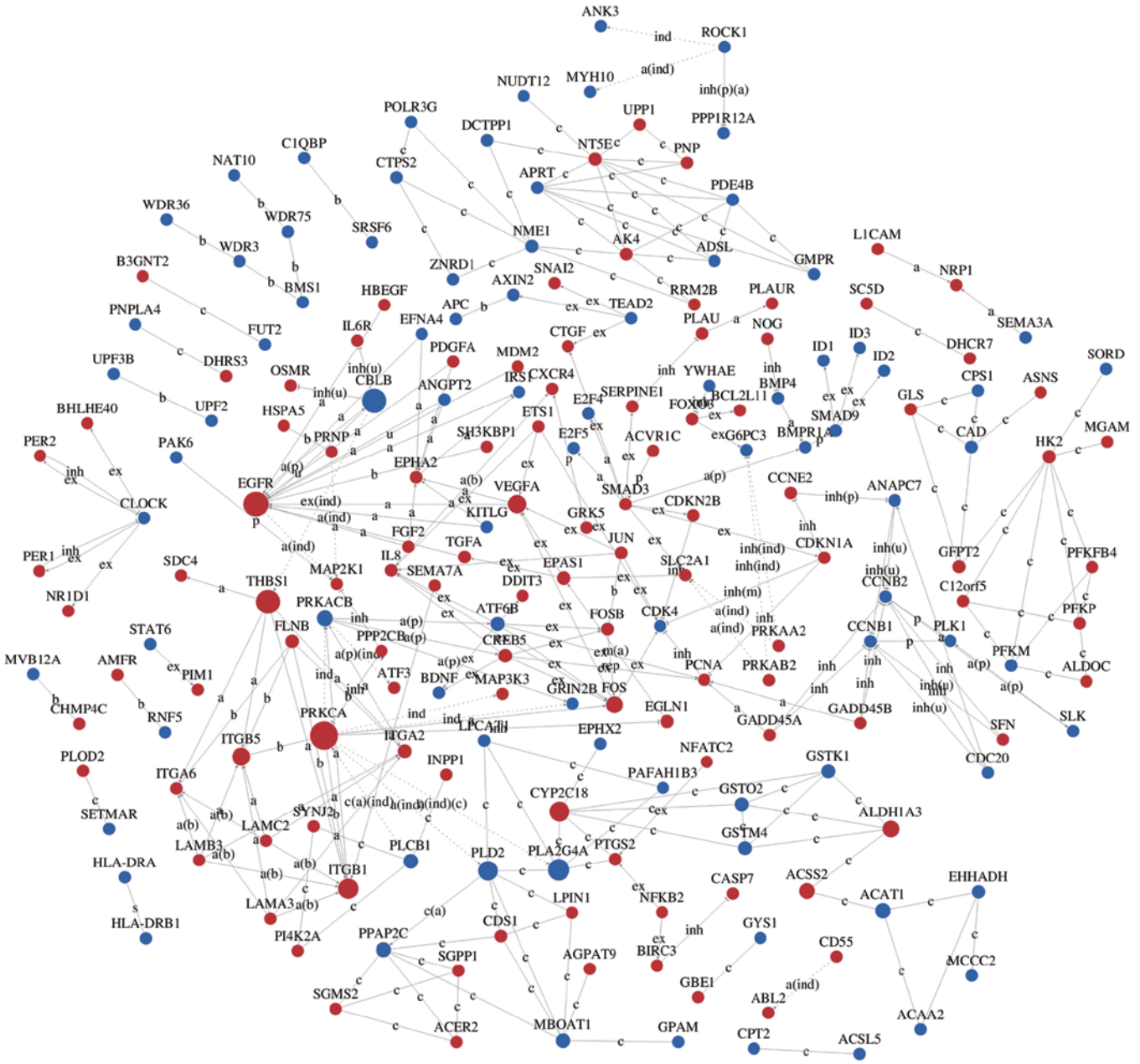

Figure 5. Gene-gene interaction network of HCT-116 cells after EA treatment. The area of circles is proportional to the value of betweenness centrality. Line segments indicate gene-gene interactions. Red indicates upregulated genes and blue indicates downregulated genes.

Discussion

Chemoprevention is emerging as an effective method for inhibiting cancer cells. Many plant polyphenols exhibit substantial inhibitory activity against the growth of colon cancer cells *in vitro* and against colon carcinogenesis in animal models (29). EA is regarded as one of the most promising and practical chemopreventive agents against various cancers (30). A previous study showed that the cytotoxicity and anti-proliferative activity of EA against cancer cells was detected at a concentration range that did not affect normal cell viability (31). *In vitro* and *in vivo* experiments have revealed that EA elicits substantial inhibitory effects against CRC, which suggests that edible EA may be of value in treatment or prevention of CRC (32-34). However, the molecular mechanisms at the protein and transcriptional levels involved in the cellular response to EA are not yet completely understood. Therefore, it is important to reveal the targets and molecular mechanisms of EA induced inhibition of CRC cell growth. To this end, we used microarray profiling, which has provided remarkable insights in many areas of modern medical research (35). The colon adenocarcinoma cell HCT-116 is widely investigated as a reliable model to check their anti-proliferative properties for various drugs (36,37). The growth inhibitory effects of EA on colon cancer cells have been previously reported at concentrations ≥100 μM *in vitro* studies (36). The present study showed that IC_{50} of EA on HCT-116 cells was 90.20 μM (data not shown). Thus, we chose 100 μM as the treatment concentration. In addition, based on literature, 72 h after the treatment is the time-point

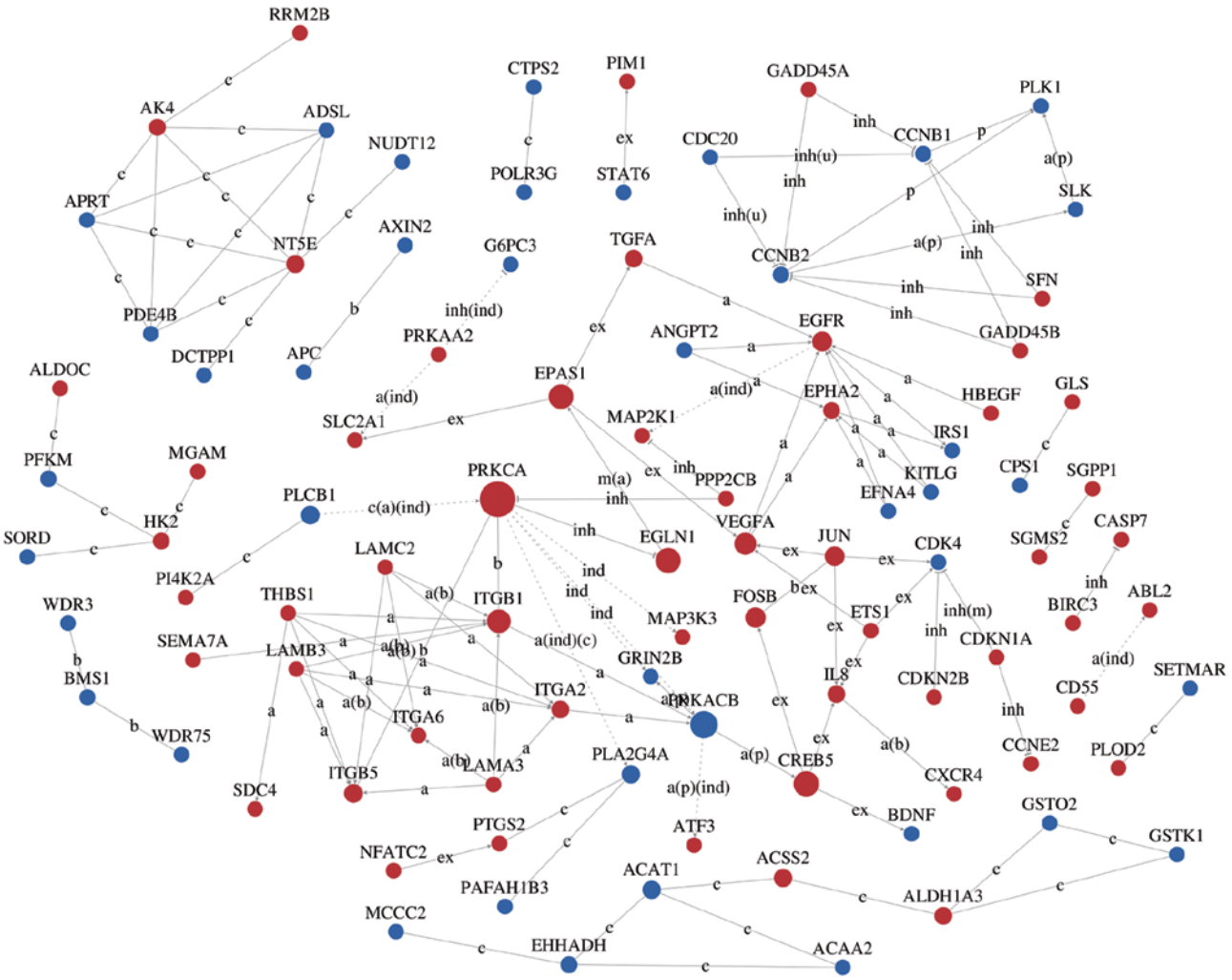

Figure 6. Regulation of proliferation, apoptosis and the cell cycle gene-gene interaction networks of HCT-116 cells after EA treatment.

often used for microarray experiment of antitumor drugs, including the EA, by evidence of significant changes of gene expression and morphology (35,38). Preliminary screening for DEGs identified 857 genes (494 upregulated and 363 downregulated) in HCT-116 cells after 72 h of exposure to EA, which are the colon adenocarcinoma cells widely investigated as a reliable model to check their anti-proliferative properties for various drugs. Among the top 10 DEGs in HCT-116 (Table II), Carboxypeptidase 4 (CPA4) is a zinc-dependent metallocarboxypeptidase on chromosome 7q32 in a region linked to prostate cancer aggressiveness. CPA4 is involved in the histone hyperacetylation pathway and may affect the growth and regulation of prostate epithelial cells (39). Centrosome-associated protein E (CENPE), a kinesin-like motor protein that accumulates in the G2 phase of the cell cycle, selectively leads to proliferation inhibition of basal-like breast cancer cell lines when inhibited. A study suggested that CENPE may be an effective therapeutic target for patients with triple-negative/basal breast cancer (40). At present, the relationships between the top 10 DEGs and the other common DEGs in the KEGG pathway database are rarely reported. Thus, they are not emerging from the results of the DEG interaction networks, making these genes important targets for our future research.

GO category analysis (Fig. 3) is becoming a standard procedure following many high throughput experimental studies, and it suggests novel hypothesis for follow-up works (41). Many key targets in CRC development and progression were identified as significantly regulated by EA. According to the P-values of each GO category, we found that the three most important functions regulated by EA were apoptotic process, cell proliferation and cell cycle arrest.

KEGG pathway analysis is the significant analysis of pathways in which these DEGs participate. Our results demonstrated 24 regulated pathways by EA, most of which are related to the apoptotic process, cell proliferation, or cell cycle arrest. EA can activate the PI3K/Akt pathway, which modulates Bcl-2 family proteins leading to an induction of apoptosis (42). EA also arrests the cell cycle of Caco-2 cells at the S- and G2/M-phases through regulation of key genes in the MAPK pathways including EGFR, KRAS, MYC, FOS and CCNB1 (42).

According to the results described above, we constructed gene-gene interaction networks (Figs. 5 and 6) to investigate the relationships among the groups of genes. The results indicate that some established key genes play important roles in the mediation of the effects of EA treatment, including IL8, ETS1, JUN and CCNB1, which are involved in cell cycle arrest (43). IL8 is a chemokine which acts on a common receptor, CXCR3,

Table III. Relative changes in expression (ratio treated/control cells) of selected 10 genes involved in proliferation, apoptosis and cell cycle in HCT-116 cells after exposure to EA for 72 h as determined by Affymetrix microarray vs. RT-PCR.

Gene symbol	Gene description	Main functions	Main related pathways	Fold change (microarray)	P-value (microarray)	Fold change (RT-PCR)	P-value (RT-PCR)
IL8	Interleukin 8	Negative regulation of cell proliferation	Pathways in cancer	2.89283	8E-05	10.3243	8E-04
JUN	Jun proto-oncogene	Negative regulation of cell proliferation	MAPK signaling pathway	2.499311	0.0003	2.72681	0.087
BCL2	B-cell CLL/lymphoma 2	Apoptosis	Apoptosis	1.327642	0.0074	1.66086	0.019
BAD	BCL2-associated agonist of cell death	Apoptosis	Apoptosis	1.034194	0.5627	1.05139	0.628
MYC	Myelocytomatosis viral oncogene homolog (avian)	Cell cycle/apoptosis	Cell cycle	-1.26086	0.0211	1.32975	0.008
CDC20	Cell division cycle 20	Mitotic cell cycle	Cell cycle/viral carcinogenesis	-2.011034	0.0007	-1.87441	0.011
IRS1	Insulin receptor substrate 1	Positive regulation of cell proliferation	PI3K-Akt signaling pathway	-2.107854	0.0016	-3.15826	0.002
CCNB1	Cyclin B1	Mitotic cell cycle/cell division	p53 signaling pathway/cell cycle	-2.149418	0.0004	-3.40518	0.004
SMC3	Structural maintenance of chromosomes 3	Mitotic cell cycle	Cell cycle	-2.16975	0.0012	-3.58037	0.006
PLK1	Polo-like kinase 1	Negative regulation of apoptotic process	Cell cycle	-2.413056	0.0004	-2.63428	0.018

to increase cell migration (44). EA also reduced the levels of CCNB1 protein, which is involved in the control of the G2/M transition and mitosis in CRC cells (42,45). Moreover, EA can inhibit cell proliferation in bladder cancer by downregulation of c-Jun, a subunit of activation protein 1 (46). However, since signaling pathways are interactive and complex, modulation of a single target is not always effective in cancer prevention and multi-targeted therapy is expected to improve treatment effectiveness. Further detailed analyses demonstrate that EA suppression of colon cancer HCT-116 cells is through simultaneous regulation of the expression of functional cancer target genes, including PRKACB, CCNB1, CDC20, JUN, MEF2C and IL8. Most of them are interacting and involved in the apoptotic process, cell proliferation or cell cycle arrest. Protein kinase C (PKC) is critical to cell proliferation, and the anti-carcinogenic action of EA has been confirmed to downregulate PKC (47). EA also inhibits the expression of markers of angiogenesis, including IL-8, VEGF and VEGFR, in mouse xenografts of the human pancreatic cancer cell line, PANC-1 (48). Moreover, the relative expression changes (ratio treated/control) of some functional genes were confirmed by RT-PCR (Table III). It is highly likely that these are the most important key targets of EA in HCT-116 cells.

These results showed that EA may play important roles in inhibiting CRC by regulating multiple targets and modulating key signaling pathways and fundamental cell processes. The results of the microarray analysis also implicate the immune response, DNA replication, and metabolism in responding to EA. A recent study found that EA induced cancer cell death by blocking energy metabolism (49). EA also significantly reduced the proliferation of human osteogenic sarcoma (HOS) cells by degrading chromosomal DNA (50). These altered functions will be the subject of our future research.

In conclusion, this study provided preliminary evidence of the antitumor effects of EA treatment on CRC cells. Microarray profiling demonstrated multiple effects of EA and provided a number of avenues for further research. Based on the results of microarray, further studies are needed to validate the multiple functions of EA and provide evidence to support its application in prevention and therapies for human CRC.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (nos. 81372612 and 81302059), the Foundation of Heilongjiang Educational Committee (no. 12541300) and the Study Abroad Returnees Science Foundation of Heilongjiang (no. LC2013C35).

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