Phellinus gilvus-derived protocatechualdehyde induces G0/G1 phase arrest and apoptosis in murine B16-F10 cells

SHI ZHONG[1], QINSHEN JIN[2], TAIHEN YU[3], JIANXUN ZHU[1] and YOUGUI LI[1]

[1]Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang 310021;
[2]Nanxun District Agricultural Technology Extension Service Center, Huzhou, Zhejiang 313009;
[3]Department of Radiology, Zhejiang Provincial People's Hospital, Affiliated People's Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China

Received April 25, 2019; Accepted November 18, 2019

DOI: 10.3892/mmr.2019.10896

Abstract. Protocatechualdehyde (PCA) is considered to be the main phenolic component of *Phellinus gilvus* responsible for its anticancer properties. Previous studies have demonstrated that PCA can have an anticancer effect on multiple cancer types, but little is known about the effect of PCA on melanoma cells. The present study investigated the inhibitory abilities and potential anticancer mechanisms of PCA on B16-F10 cells using MTT assay. Cell apoptosis and cell cycle were assessed by flow cytometry using Annexin V-FITC and propidium iodide staining. Whole-transcriptome analysis was used to investigate the effects of PCA on gene expression. PCA significantly decreased cell viability, induced cell cycle arrest at G0/G1 phase and promoted apoptosis of B16-F10 cells, suggesting that PCA could have anticancer effects against melanoma cells. Whole-transcriptome analysis indicated that PCA treatment upregulated genes involved in histone modification and decreased the transcription of genes involved in DNA repair and replication. Kyoto Encyclopedia of Genes and Genomes analysis showed that PCA treatment enhanced the complement and coagulation cascades, and the p53 signaling pathway. The present results indicated that PCA could act as an antitumor agent in melanoma cells, which may provide experimental support for the development of novel therapies to treat melanoma.

Introduction

Melanoma is a common type of skin cancer, causing >30% of skin cancer-related mortalities, despite accounting for <4% of total skin cancer types in the US (1). The incidence of melanoma has increased by ~4% in the past decade (2). Although treatment of melanoma has improved, finding effective therapeutics with low toxicity is urgent (3). In this regard, herbal and dietary medicines have been considered a promising alternative for melanoma therapy (4). Previous studies have tested the anti-melanoma activities of natural products isolated from fruits, vegetables and plants (5). Herb-derived polyphenols have been intensively investigated due to their abilities to reduce the aggressiveness of melanoma (6,7).

Protocatechualdehyde (PCA) is a polyphenol compound found in many Chinese herbal plants, such as *Salvia miltiorrhiza*, *Stenoloma chusanum* (L.) Ching and *Ilex chinensis* Sims (8,9). PCA can also be isolated from the butanol extract of *Streptomyces lincolnensis* M-20 (10), and is thought to be the main phenolic component of *Phellinus gilvus* responsible for its antioxidant effect (11). PCA has anticancer effects against multiple cancer types, including breast cancer (10), leukemia (12) and colorectal cancer (11). Our previous study reported that *P. gilvus*-derived PCA inhibited the proliferation of human colorectal cancer cells by inducing S phase arrest and promoting the mitochondrial apoptotic pathway (11). Whilst the cytotoxic properties of PCA against cancer cells have previously been shown (11), little is known about the effect and mechanism of PCA in melanoma cells.

The present study investigated the cytotoxic activity of *P. gilvus*-derived PCA in B16-F10 cells. Whole-transcriptome sequencing was used to evaluate PCA-induced differentially expressed mRNAs to identify the molecular mechanisms by which PCA may inhibit melanoma development.

Materials and methods

Chemicals and reagents. Murine melanoma B16-F10 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS (both purchased from Gibco; Thermo Fisher Scientific, Inc.), plus penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cells were cultured at 37˚C under a humidified atmosphere with 5% CO_2. MTT was obtained from Sigma-Aldrich (Merck KGaA). Apoptosis assay kit was purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Correspondence to: Dr Yougui Li, Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences, 298 Desheng Road, Hangzhou, Zhejiang 310021, P.R. China
E-mail: liyougui3@126.com

Key words: protocatechualdehyde, melanoma, B16-F10 cells, apoptosis, p53 signaling pathway

PCA preparation. PCA was isolated from the fresh fruiting bodies of *Phellinus gilvus*, which was cultivated by Sericultural Research Institute (Zhejiang Academy of Agricultural Sciences). The purification and identification of PCA was performed as previously described (11). High-performance liquid chromatography (HPLC) was performed on a 1260 Infinity II LC System (Aligent Technologies, Inc.). The separation was performed on an Agilent ZORBAX Eclipse Plus-C18 (100x4.6 mm; 1.8 μm; Aligent Technologies, Inc.). The binary mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was kept constant at 0.6 ml/min for a total run time of 50 min, and the column temperature was maintained at 30˚C. The system was run with a gradient program: 0-30 min, 3-30% A; 30-50 min 30-95% A. The sample quantity was 20 μl. HPLC analysis showed that the purity of PCA was >95%.

Cell proliferation assay. Cell proliferation was determined by an MTT-based colorimetric assay. Cells at the exponential growth phase were harvested and dispensed into a 96-well microplate at 100 μl/well. After 24 h, 100 μl PCA was added to different final concentrations (0, 0.5, 1, 2.5, 5, 12.5, 25, 50, 100 and 200 μg/ml) and incubated at 37˚C for 24, 48 or 72 h. Cells were then incubated at 37˚C with 50 μl of MTT solution (1 mg/ml) for 2 h and the resulting crystals were dissolved in DMSO. The absorbance at 570 nm was recorded to assess the formation of formazan.

Cell cycle and apoptosis analysis by flow cytometry. The cell cycle phase was measured by assessing the DNA content using flow cytometry. B16-F10 cells ($2x10^4$ cells/well) were incubated at 37˚C with PCA at different final concentrations of 0, 12.5 or 50 μg/ml for 48 h. Cells were collected and washed with ice-cold PBS, and fixed at 4˚C in 70% ethanol for 12 h. After staining with propidium iodide (PI; 0.5 ml) at 4˚C for 30 min, the proportion of cells at different phases was determined using a flow cytometer (Cytomics FC 500 MCL; Beckman Coulter, Inc.) and MultiCycle AV software (CXP V2.3 WIN7, C30309; Phoenix Flow Systems, Inc.) was used for analysis. Apoptosis rate was measured by staining at 4˚C for 15 min with Annexin V-FITC (5 μl) and PI (5 μl). Annexin V^-/PI^+ (lower right) cells were deemed as early apoptotic cells, Annexin V^+/PI^+ (upper right) cells were defined as late apoptotic cells, while Annexin V^+/PI^- (upper left) cells were necrotic cells. All experiments were performed in triplicate.

RNA sequencing. After treatment with PCA (50 μg/ml) and 0.1% DMSO (Control) for 48 h, B16-F10 cells ($1x10^7$) were collected for RNA sequencing. Total RNA was obtained from cells using a RNAsimple Total RNA kit (Biotech Co., Ltd.). The construction of cDNA libraries and RNA-Seq was performed by Guangzhou RiboBio Co., Ltd. The HISTAT2 was used to map clean reads to the genome with the default parameters (13). The threshold value of differentially expressed mRNA was set by $|\log_2 FoldChange|>1$ and q-value <0.02.

Bioinformatics analysis. Principal component analysis was used to assess the differences between groups and the duplication within groups, according to the expression level of all genes in each sample. DEseq2 was used to analyze the differential expression of genes in the control group and PCA-treated B16-F10 cells (14). Gene Ontology (GO) term enrichment analysis of differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (15). KOBAS (version 2.0, http://kobas.cbi.pku.edu.cn/) was used to test the statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (16,17).

RT-quantitative (q)PCR analysis. Total RNA extraction, cDNA synthesis and RT PCR were performed as previously described (11). Briefly, total RNA was isolated from B16-F10 cells treated with or without PCA (50 μg/ml) for 48 h using the TaKaRa MiniBEST Universal RNA Extraction kit (Takara Bio, Inc.), then the cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc.). RT-qPCR was performed using the SYBR® Fast qPCR Mix (Takara Bio, Inc.) via the StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). The PCR volume was 20 μl, including 0.4 μl each primer (10 nmol/l), 10 μl SYBR Fast qPCR Mix, 0.4 μl ROX reference dye, 2.0 μl cDNA template and 6.8 μl dilution buffer (Takara Bio, Inc.). An initial activation at 95˚C for 2 min was followed by an amplification target sequence of 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. PCR primers were synthesized by Shanghai Shenergy Biocolor BioScience & Technology Co., Ltd. as follows: p53-induced death domain-containing protein 1 (PIDD) forward 5'-GGGAACCAGTTGAACTTG GAC-3', reverse 5'-CTGAGCCGCAAAAACTCCAC-3'; p21 forward 5'-CCTGGTGATGTCCGAC-CTG-3', reverse 5'-CCA TGAGCGCATCGCAATC-3'; Cyclin E forward 5'-TCT-TCA CACAGATGACACAAGC-3', reverse 5'-CAACAGCAACCT ACAACACCC-3'; Bax forward 5'-AGACAGGGGCCTTTT TGCTAC-3', reverse 5'-AATTCGCCG-GAGACACTCG-3'; Cyclin D forward 5'-TCAAGTGTGACCCGGACTG-3', reverse 5'-ATGTCCACATCTCGCACGTC-3'; growth arrest and DNA damage-inducible 45 (Gadd45) forward 5'-CCGAAAGGA TGGACACGGTG-3', reverse 5'-TTATCGGGGTCTACGTTG AGC-3'; p53 forward 5'-CACAGCACATGACGG-AGGTC-3', reverse 5'-TCCTTCCACCCGGATAAGATG-3'; Bcl-2 forward 5'-GCTACCGTCGTGACTTCGC-3', reverse 5'-CCCCAC CGAACTCAAAGAAGG-3'; cell division cycle-associated protein 2 (Cdc-2) forward 5'-AGAAGGTACTTACGG-TGT GGT-3', reverse 5'-GAGAGATTTCCCGAATTGCAGT-3'; Cyclin B forward 5'-AAGGCCAAGGTCAGTATGGC-3', reverse 5'-CTTGCCTGTAGCTCTTCGCT-3'; phosphatidylinositol glycan anchor biosynthesis class S (PIGs) forward 5'-TCT-GTCCCCGATTTCCCCC-3', reverse 5'-AGCTGCTTC AAGACTTCCGC-3'; GAPDH forward 5'-AAGAAGGTG GTGAAGCAGG-3', reverse 5'-GAAGGTGGA-AGAGTG GGAGT-3'. The PCR product sizes for PIDD, p21, cyclin E, Bax, cyclin D, Gadd45, p53, Bcl-2, Cdc-2, cyclin B, PIGs and GAPDH were 107, 103, 88, 137, 175, 121, 101, 147, 128, 131, 125 and 111 bp, respectively. Gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (18).

Statistical analysis. Data are expressed as the mean ± SEM. Statistical analysis was performed using the SPSS 16.0 (SPSS, Inc.). One-way ANOVA was used to analyze statistical differences between groups under different conditions,

followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

PCA inhibits the viability of B16-F10 melanoma cells. To examine the cytotoxicity of PCA on the proliferation of melanoma cells, B16-F10 cells were treated with different concentrations of PCA (0, 0.5, 1, 2.5, 5, 12.5, 25, 50, 100 and 200 μg/ml) for 24, 48 and 72 h, and MTT assay was performed. Treatment with PCA for 24, 48 and 72 h significantly decreased cell viability compared with the control group (PCA 0 μg/ml) in a dose-dependent manner (Fig. 1).

At higher concentration levels (12.5, 25, 50, 100 and 200 μg/ml), PCA significantly decreased the growth of B16-F10 cells. The present results suggested that extended treatment periods with PCA could, to a greater extent, reduce B16-F10 cell growth. These results suggested that PCA could be used as a cell proliferation inhibitor for the treatment of melanoma.

PCA induces cell cycle arrest at the G0/G1 phase. Flow cytometry was used to assess whether the antiproliferative effects of PCA were mediated by the arrest of the cell cycle in B16-F10 cells treated with 0, 12.5 or 50.0 μg/ml PCA for 48 h. PCA significantly increased cell number at the G0/G1 phase and decreased cell number at the S phase in a dose-dependent manner (Fig. 2). The influence of PCA on the cell number at the G2/M phase was not clear in the low dose PCA treatment group (12.5 μg/ml), but a significant increase was observed in the high dose PCA-treated group (50.0 μg/ml). The present results suggested that G0/G1 phase arrest may be partially involved in the mechanism of PCA-reduced viability of B16-F10 cells.

PCA promotes apoptosis in B16-F10 cells. Flow cytometry with Annexin V-FITC and PI staining was used to examine whether apoptosis was involved in the PCA-mediated growth inhibition of B16-F10 cells. PCA significantly increased the number of early apoptotic, late apoptotic and necrotic cells in a dose-dependent manner (Fig. 3). Treatment with PCA significantly increased early and late apoptosis. Treatment with 50 μg/ml PCA increased the percentage of early and late apoptotic cells by 54- and 25-fold, respectively.

PCA affected the transcriptome of B16-F10 cells. RNA sequencing was performed to monitor the gene expression profile of PCA-treated B16-F10 cells. Principal component analysis (Fig. 4A) showed that PCA-treated cells were completely separated from the vehicle-treated control cells (P<0.01), indicating that PCA affected the whole gene expression of B16-F10 cells. In total, 3,578 differentially expressed genes were identified (Fig. 4B). Among them, 1,820 genes were upregulated, and 1,758 genes were downregulated. The differentially expressed genes between the PCA-treated and the control group were clustered (Fig. 4C) and further classified into biological process (BP), cellular components (CC) and molecular function (MF) according to standard GO terms (Fig. 4D and E). In the BP category, the genes that were upregulated by PCA treatment were found to

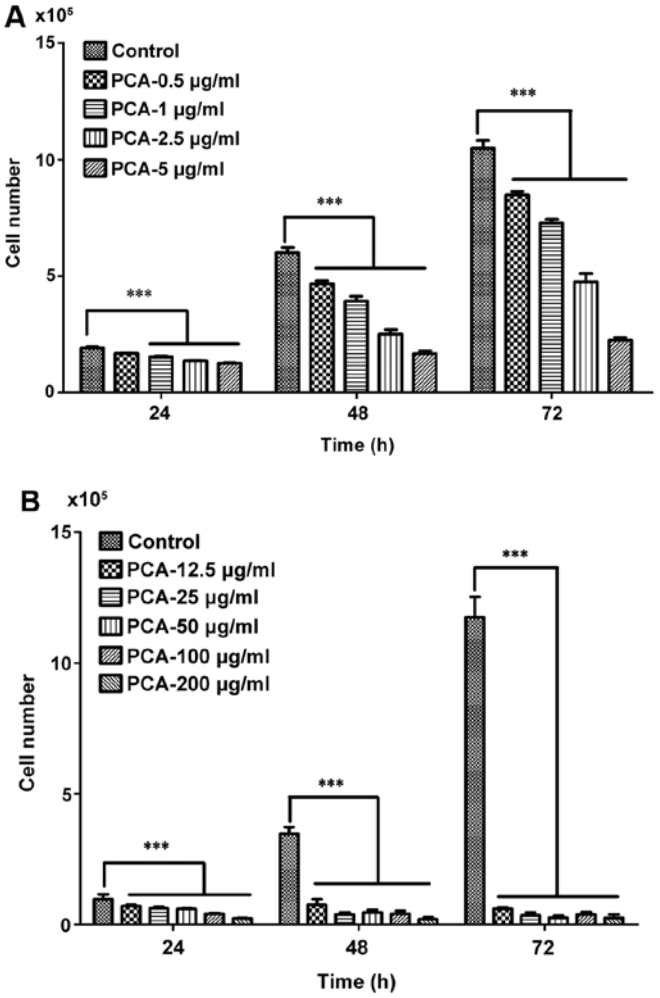

Figure 1. PCA inhibits the viability of B16-F10 cells. B16-F10 cell viability was assessed by the MTT assay over time (0, 24, 48 and 72 h) and with increasing concentrations of PCA at (A) 0.5, 1, 2.5, 5 μg/ml and (B) 12.5, 25, 50, 100 and 200 μg/ml; 0 μg/ml of PCA was the control. All experiments were performed in triplicate. ***P<0.001 vs. control group. PCA, protocatechualdehyde.

be involved in cellular processes, such as histone trimethylation, peptidyl-lysine trimethylation and histone modification (Fig. 4D), while downregulated genes were found to be associated with DNA repair and DNA replication (Fig. 4E). In the CC category, PCA primarily affected the expression levels of genes located in the extracellular region or on the cytoskeleton compared with the control cells (Fig. 4D), while PCA decreased the expression of mitochondrial components (Fig. 4E). Under the MF category, PCA treatment mainly led to the upregulation of genes associated with receptor binding (Fig. 4D) and the downregulation of genes involved in DNA damage and protein binding (Fig. 4E).

Analysis using the KEGG database indicated that PCA may inhibit the growth of B16-F10 cells via the following pathways: i) 'Complement and coagulation cascades'; ii) 'p53 signaling pathway'; and iii) 'regulation of actin cytoskeleton' (Fig. 4F). Detailed analysis of the p53 signaling pathway showed that PCA could upregulate multiple genes involved in this pathway (Fig. 5) (19). Furthermore, the expression of p53-related genes in B16-F10 cells was investigated using RT-qPCR (Fig. 6). The present results showed that PCA significantly increased

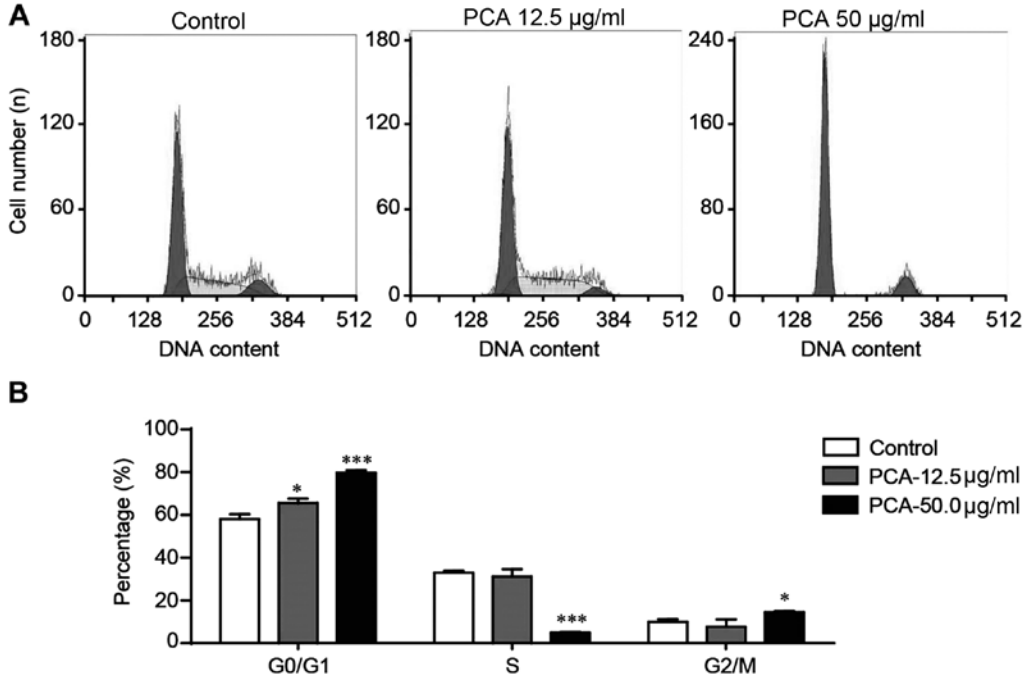

Figure 2. PCA induces cell cycle arrest at G0/G1 phase. (A) Flow cytometry with propidium iodide staining. (B) Percentage of cells at different cell cycle phases. B16-F10 cells were treated with different concentrations of PCA for 48 h and then cell cycle was tested by flow cytometry. All experiments were performed in triplicate. *P<0.05, ***P<0.001 vs. vehicle control group. PCA, protocatechualdehyde.

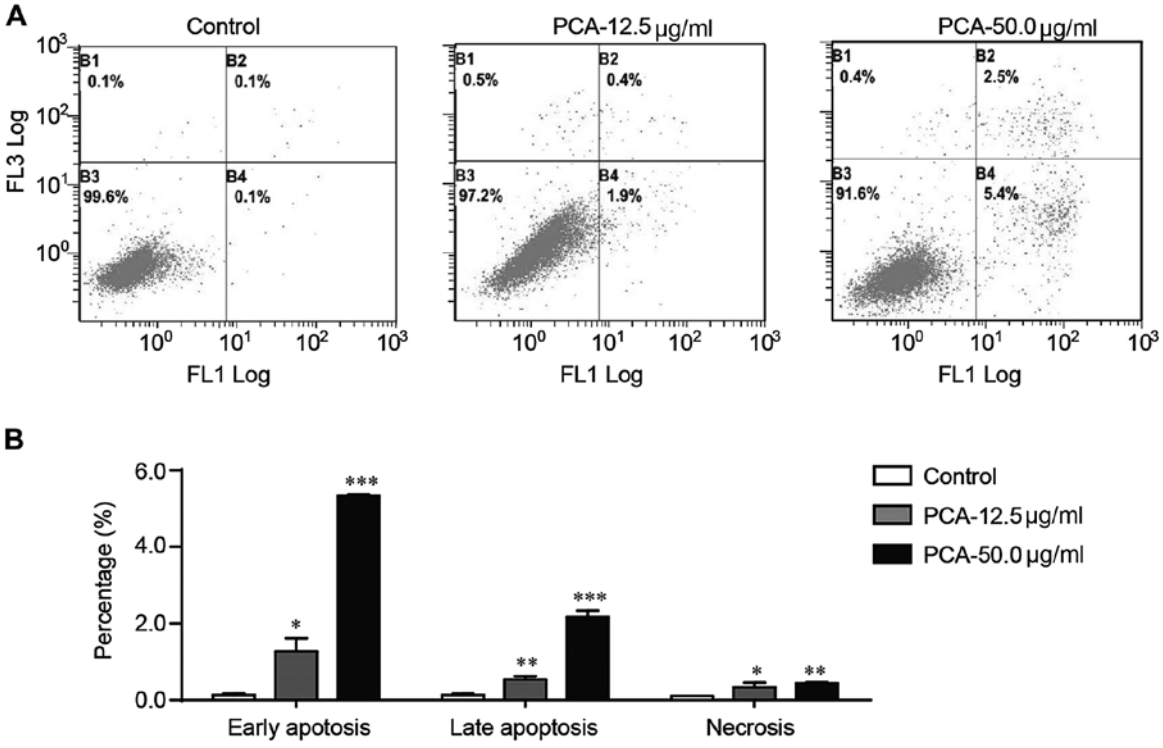

Figure 3. PCA induces apoptosis. (A) Cell apoptosis indicated by Annexin V-FITC/propidium iodide staining after PCA treatment. (B) Percentage of cells at early apoptosis, late apoptosis and necrosis stages. B16-F10 cells were treated with different concentrations of PCA for 48 h and then apoptosis was measured by flow cytometry. All experiments were performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle control group. PCA, protocatechualdehyde.

the expression level of p21 and PIDD (Fig. 6A), but significantly downregulated the expression of cyclin D, Gadd45, p53, Bcl-2, Cdc-2, cyclin B and PIGs after 48 h exposure compared with the control (Fig. 6B). However, no detectable changes in cyclin E and Bax expression were observed after PCA treatment (Fig. 6A). Therefore these findings suggested that PCA could regulate the p21/cyclin D/CDK4/6 pathways, which may induce G1 phase arrest.

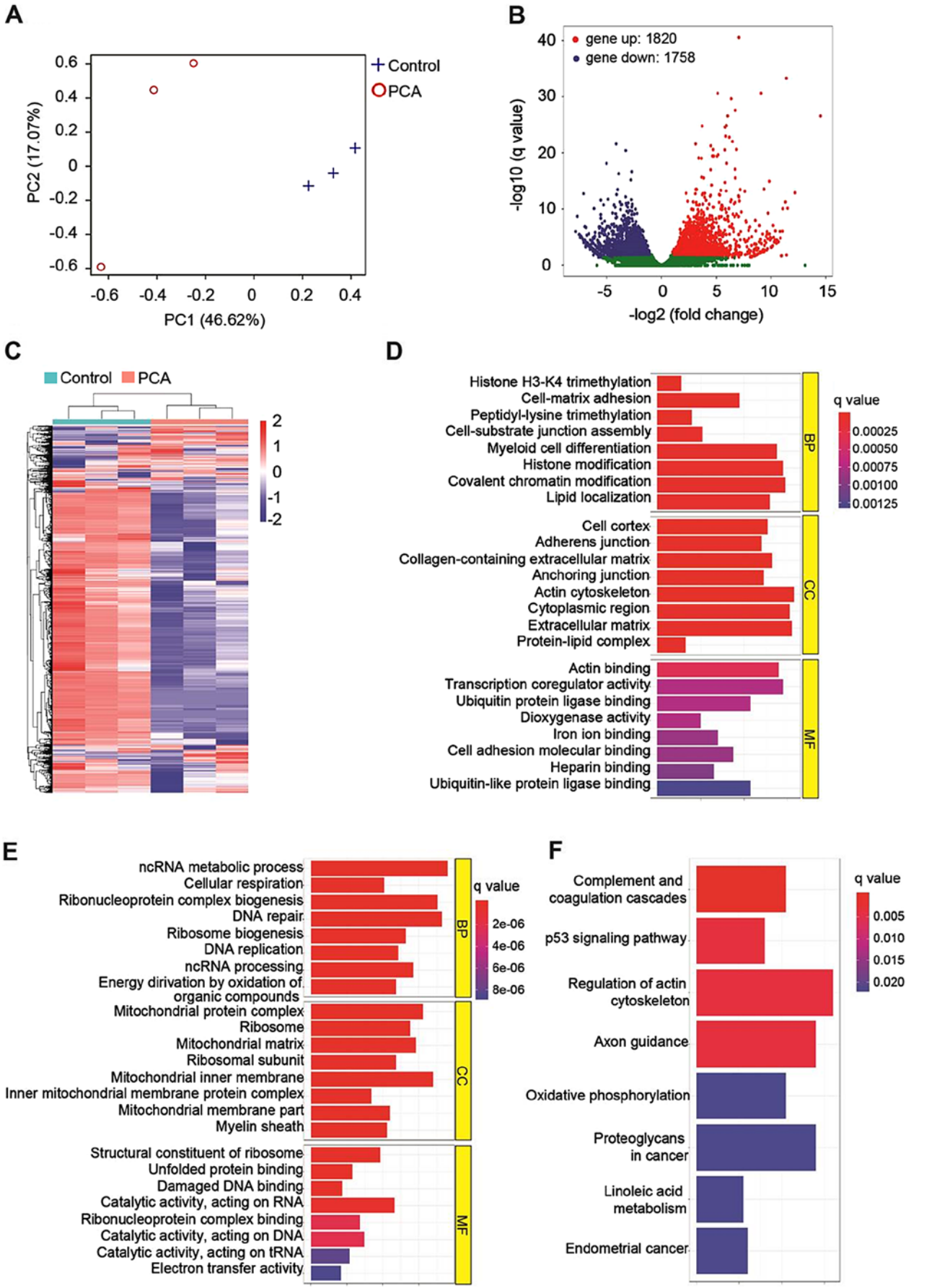

Figure 4. Statistically significant differentially expressed genes regulated by PCA. (A) Principal component analysis. (B) Volcano Plot and (C) heatmap of the differentially expressed mRNAs. Gene Ontology analysis of the significantly (D) upregulated and (E) downregulated genes in BP, CC and MF. (F) Kyoto Encyclopedia of Genes and Genomes pathway analysis of the upregulated pathways following PCA treatment. PCA, protocatechualdehyde; BP, Biological Process; CC, Cellular Component; MF, Molecular Function; PC, principal component.

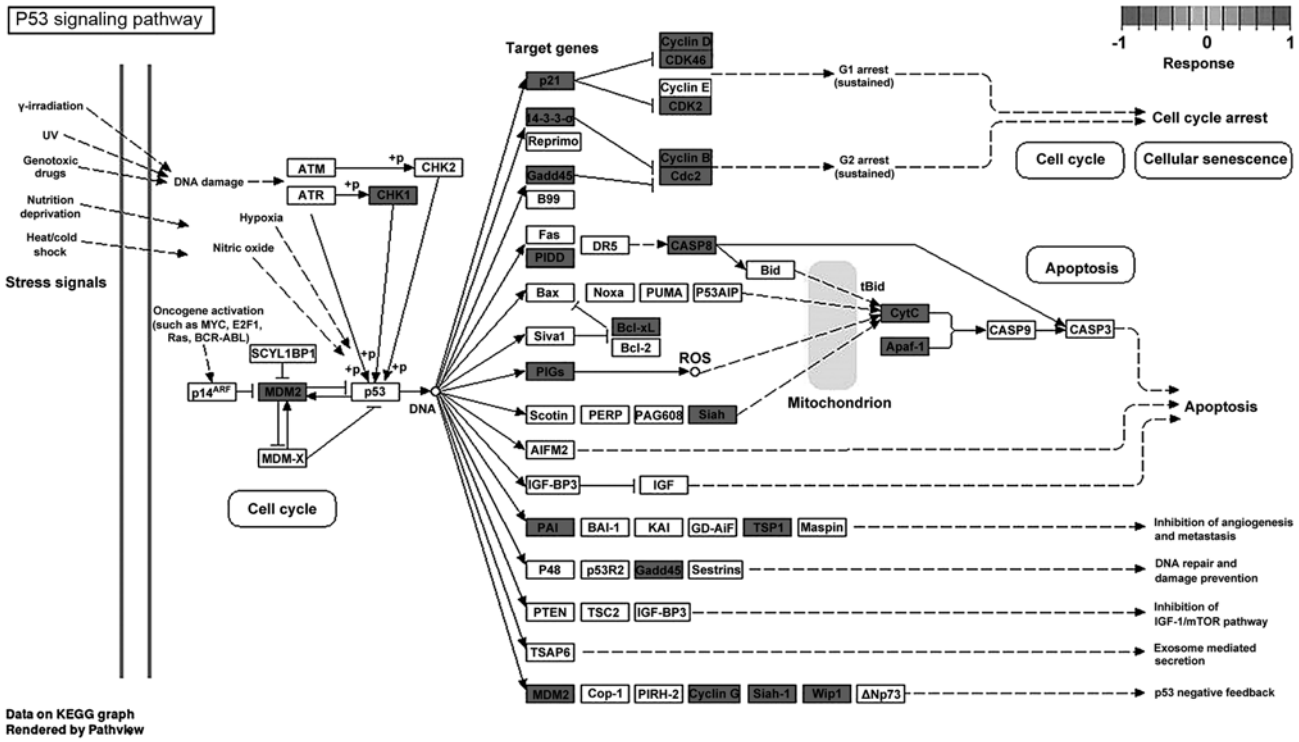

Figure 5. KEGG pathway analysis of the p53 signaling pathway regulated by PCA. Copyright permission was granted by Kanehisa Laboratories to adapt a KEGG pathway map for this figure. PCA, protocatechualdehyde.

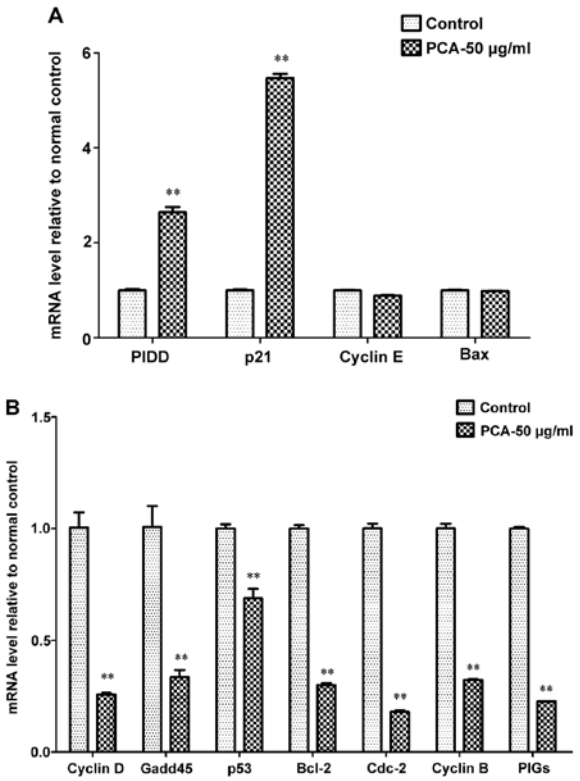

Figure 6. mRNA expression analysis of p53-related genes in B16-F10 cells treated with and without 50 μg/ml PCA for 48 h. mRNA expression level of the following genes: (A) PIDD, p21, cyclin E and Bax; and (B) cyclin D, Gadd45, p53, Bcl-2, Cdc-2, cyclin B and PIGs. Density values were normalized to GAPDH. Data are presented as the mean ± SD from three experiments per group. **P<0.01 vs. control. PCA, protocatechualdehyde; Gadd45, growth arrest and DNA damage-inducible 45; PIDD, p53-induced death domain-containing protein 1; Cdc-2, cell division cycle-associated protein 2; PIGs, phosphatidylinositol glycan anchor biosynthesis class S.

Discussion

Natural products offer a source for the identification of novel drugs for the treatment of various diseases, including cancer. In total, >50% of pharmaceutical drugs can be traced back to natural compounds or their analogs (20). Previously, chemoprevention via the dietary consumption of natural products has gained considerable attention due to their low toxicity, patient acceptance and effectiveness (21). Polyphenols are a large and complex family of organic compounds that are present in various plants and fungi (22). Polyphenols could be used as potential anticancer agents due to their antioxidant, anti-inflammatory, antiproliferative and anticarcinogenic properties (23). In the present study PCA, a natural polyphenol compound, was isolated from the medical mushroom *P. gilvus*. A previous study showed that PCA can exert antiproliferative effects in several cancer cell lines (11). Our previous study demonstrated that PCA inhibits the viability of human colorectal cancer cells by inducing S phase arrest and promoting the mitochondrial apoptotic pathway (11). However, to the best of our knowledge, whether PCA could impact melanoma development has not been previously reported so the present study is the first to report that PCA could exert a growth inhibitory effect on murine melanoma cells by inducing G0/G1 phase arrest and promoting apoptotic cell death.

The cell cycle is strictly regulated by a complex network of events that promote or stop the progression of cells from one phase to the other (24). p21 plays a crucial role in cell cycle progression and cancer (24); it is able to mediate p53 activity and promote cell cycle arrest by inhibiting the activity and formation of cyclin/CDK complexes (24). At high levels,

p21 inhibits the function of CDKs (25), thus inhibiting the cyclin D/CDK4/6 complex, resulting in G1/S arrest (26). In the present study, treatment with PCA significantly increased the number of cells in the G0/G1 phase and decreased the number of cells in the S phase, which arrested the cell cycle of melanoma cells at the G0/G1 phase. Our previous results showed that PCA inhibited the proliferation of HT-29 cells and induced cell cycle arrest in the S phase by downregulating the expression of cyclin A and D (11). In the present study, PCA significantly increased p21 mRNA expression level and downregulated Cyclin D expression in B16-F10 cells, suggesting that PCA inhibited B16-F10 cell proliferation and blocked entry to the S phase by regulating the p21/Cylin D/CDK4/6 pathway.

Apoptosis is a main mechanism by which anticancer drugs can suppress tumor growth (27). p53 plays a key role in promoting cell cycle arrest or apoptosis in response to acute DNA damage (28). This p53-induced cell cycle arrest allows the damaged DNA to be repaired, thereby preventing the proliferation of cancer cells (28). Anticancer agents can trigger apoptosis, by either directly inducing DNA damage or indirectly inducing secondary stress-responsive signaling pathways, which leads to the activation of the intrinsic mitochondrial apoptotic pathway (29). The Bcl-2 family consists of pro- and anti-apoptotic structurally related proteins that are principal regulators of the mitochondrial apoptotic pathway (29). Anti-apoptotic protein Bcl-2 acts as a gatekeeper that inhibits the release of cytochrome *c* from the mitochondria to the cytoplasm (29). In the present study, flow cytometry analysis showed that PCA significantly promoted apoptosis, and significantly increased the number of early apoptotic cells (54-fold) and late apoptotic cells (25-fold). Whole-transcriptome analysis further indicated that PCA decreased the expression levels of the genes participating in DNA repair and replication. Furthermore, RT-qPCR results showed that PCA markedly decreased the expression of Bcl-2, indicating that it may trigger apoptosis via the mitochondrion-dependent pathway. The present findings suggested that PCA stimulated cell cycle arrest and apoptosis via upregulation of the p53 signaling pathway.

In conclusion, the present study suggested that the *P. gilvus*-derived polyphenol compound PCA could effectively inhibit the growth and proliferation of melanoma cells by inducing G0/G1 phase arrest and promoting apoptotic cell death. These cellular changes may be mediated by the activation of the p53 signaling pathway.

Acknowledgements

Not applicable.

Funding

This work was supported financially by The Science and Technology Department of Zhejiang Province (grant nos. 2018C02003 and LGN18C170005), Zhejiang Medical and Health Science and Technology Project (grant no. 2018KY250) and Key Laboratory of Creative Agriculture, Ministry of Agriculture and Rural Affairs.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SZ and YL contributed to the conception of the study. SZ, QJ and JZ performed the experiments. YL contributed significantly to the data analysis and manuscript preparation. TY contributed to the data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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