

PLAGL1 Gene Demethylation Induced by Epigallocatechin Gallate Promotes Pheochromocytoma Cell Apoptosis Via Wnt/ β -catenin Signaling Pathway

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Abstract

Objective: The aim of this study was to investigate the expression and the promoter methylation level of *PLAGL1* gene and the mechanism of epigallocatechin gallate (EGCG) that induces *PLAGL1* gene demethylation and promotes the apoptosis of pheochromocytoma (PCC) in PC12 cell line. **Methods:** The PC12 cells were treated with 25, 50, 75, 100, and 150 μ g/mL EGCG for 48 hours. MSP was used to examine *PLAGL1* gene methylation and an MTT assay was performed to detect the cell proliferation. The cell apoptosis was detected using flow cytometry. The mRNA and protein expressions of DNMT1, *PLAGL1*, Wnt, and β -catenin were detected using RT-quantitative PCR and Western blot. **Results:** EGCG dose-dependently reduced the cell viability and reversed *PLAGL1* gene hypermethylation in PC12 cells ($P < 0.05$). The cell apoptosis was significantly increased in PC12 cells treated with EGCG. The EGCG treatment restored the expressions of *PLAGL1* and downregulated the expression of DNMT1, Wnt, and β -catenin in PC12 cells ($P < 0.05$). **Conclusion:** The EGCG induces the demethylation process of *PLAGL1* gene through down-regulating DNMT1 and restores the *PLAGL1* mRNA and protein expression. The Wnt/ β -catenin signaling pathway is involved in the regulation of PCC cell apoptosis promoted by EGCG inducing *PLAGL1* gene demethylation.

Keywords: Epigallocatechin gallate- *PLAGL1* gene- methylation- Wnt/ β -catenin signaling pathway

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Introduction

Pheochromocytoma (PCC) is a rare tumor derived from chromaffin cells, usually accompanied by the overgeneration of epinephrine and norepinephrine, which results in paroxysmal or sustained hypertension (Fishbein et al., 2021). The pathogenesis of PCC is associated with epigenetics changes and studies have shown that promoter CpG island hypermethylation of the tumor suppressor gene is associated with the progression and prognosis of PCC (Yeung et al., 2021). Therefore, reversing the hypermethylation status of tumor suppressor genes is expected to be a potential therapeutic strategy for PCC.

The DNA methyltransferase plays an important role in DNA methylation, while DNMT inhibitors reduce the hypermethylation status and restore the expression of tumor suppressor gene (Hu et al., 2021). However, most prevalent synthetic DNMT inhibitors lack target specificity and cause side effects such as instability and cell toxicity (Pathania et al., 2016). Compared to these

inhibitors, the small-molecule natural products become a new target epigenetic regulator with low toxicity and cost-effective benefits (Schnekenburger and Diederich, 2012). Epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, can inhibit tumor cell proliferation by inducing tumor cell apoptosis. Accumulating proofs confirm that EGCG directly inhibits the function of DNMT and reversed the methylation status of tumor suppressor genes (Gan et al., 2018; Morris et al., 2016). Sheng (2019) found that EGCG could inhibit the proliferation of breast cancer cells through the demethylation of *SCUBE2* promoter and restore the expression of *SCUBE2*. EGCG may play a targeted role in the methylation of tumor suppressor genes.

Studies have shown that the methylation rate of *PLAGL1* gene in malignant PCC tissue was higher than that in normal adrenal medulla, while the relative mRNA expression of *PLAGL1* significantly decreased in malignant PCC tissue (YANG et al., 2014). The *PLAGL1* gene was located in chromosome 6q24-q25 and

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identified as a tumor suppressor gene. PLAGL1 provides instructions for producing zinc finger proteins and acts as a transcription factor involved in inducing cell apoptosis through regulating Wnt/ β -catenin signaling pathway (Ahn et al., 2021; Zhu et al., 2009). This study is aimed at investigating the demethylation effects of EGCG in the *PLAGL1* gene and exploring whether EGCG inhibits cell proliferation through inducing the *PLAGL1* gene demethylation via regulating Wnt/ β -catenin signaling pathway in PCC.

Materials and Methods

Cell Culture

The PC12 cells were purchased from the National Collection of Authenticated Cell Cultures. The RMPI 1640 containing 10% fetal bovine serum was used to culture PC12 cells in the presence of 5% CO₂/95% air atmosphere at 37 °C. When PC12 cells fusion reached 80–90% confluency, the cells were seeded into the plates and treated with EGCG at doses of 25, 50, 75, 100, and 150 μ g/mL.

MSP assay

Genomic DNA was extracted using TIANamp genomic DNA Kit (Tiangen, China). According to the manufacturer's instructions, DNA purity was measured using the NanoDrop micro spectrophotometer (Thermo, USA). The EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) was used to modify the purified DNA. After bisulfite treatment, the modified DNA was subjected to amplification using PCR. The methylated specific upstream primer of *PLAGL1* was 5'-GTAAATTAAGGTTTCGGGGC-3'; the methylated specific downstream primer of *PLAGL1* was 5'-CCTTAACCTAACGTATTACGCGACA-3'; the unmethylated specific upstream primer of *PLAGL1* was 5'-GGTAAATTAAGGTTTGGGG-3'; and the unmethylated specific downstream primer of *PLAGL1* was 5'-CATCCCCTTAACATATTACACAAC-3'. The PCR Reactions conditions were pre-denaturation at 95 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, elongation at 72 °C for 1 min, 40 cycles Ring, then an extra elongation at 72 °C for 7 min. In addition, 2% agarose gel was prepared to detect the PCR products in the electrophoresis tank.

MTT assay

Cell viability was evaluated using a methyl thiazolyl tetrazolium (MTT) assay. The PC12 cells were suspended in 5000 cells/well and seeded into 96-well plates. The cells were incubated with different concentrations of EGCG for 48 h, the MTT (1mg/mL) was added to cells and then incubated for 4h at 37 °C. The mediums were removed and formazan was dissolved using dimethyl sulfoxide (DMSO, 150 μ L/well). Phosphate buffer saline (PBS) was used to wash the cells. Absorbance values at 570 nm were detected on a microplate reader (Thermo, USA).

Flow cytometric analysis

The PC12 cells were seeded into 6-well plates

and treated by EGCG for 48 h and then harvested and centrifuged at 1200 rpm for 5 min. Ice-cold PBS was used to wash the cells and resuspended twice. Cell apoptosis was measured by annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech, China).

RT-qPCR

The PC12 cells were inoculated into 12-well plates and treated by EGCG for 48 hours. The cells were harvested through centrifugation at 1200 rpm for 5 min, ice-cold PBS was used to wash the cells, and Trizol reagent (Takara, Japan) was used to extract the total RNA. Nanodrop micro spectrophotometer was used to analyze the RNA quality. PrimeScript RT Reagent Kit with gDNA Eraser-Takara, Japan-was used to synthesize a first strand cDNA from 2 mg of total RNA, PCR amplification was performed by SYBR Green qPCR Master Mix (Thermo, USA). The specific primer sequences are listed in Table 1. The β -actin was used to normalize the amount of mRNA and 2^{- $\Delta\Delta$ Ct} method was performed to calculate the relative gene expression.

Western Blot

The RIPA Buffer was used to lyse the PC12 cells, proteins were isolated, and BCA assay was performed to determine the total protein concentration.

Around 50 μ g protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently PVDF membranes were used to transfer the protein using tris-glycine buffer at a constant voltage. In total, 5% nonfat milk was used to block the membranes for 1h at room temperature, and then the primary antibodies were added to incubate the membranes at 4 °C overnight. Afterward, tris buffered saline with Tween-20 was used to wash the membranes three times and incubated with the secondary antibodies at room temperature for 1 h e. Protein bands were then visualized using ECL detection reagent (Thermo Fisher Scientific, USA). Densitometry was analyzed using Image Lab software (BioRad, Hercules, CA, USA).

Statistical analysis

The results are presented as the mean \pm standard error. Three separate tests were performed in triplicate. One-way analysis of variance (ANOVA) was used to estimate the statistical significance of differences in SPSS 17 ($P < 0.05$)

Results

The Methylation Level of PLAGL1 Promoter in PC12 Cells was reduced by EGCG

The methylation status of the *PLAGL1* promoter was analyzed using MSP. The DNA methylation levels in the CpG islands of *PLAGL1* was increased in PC12 cells. Comparing to PC12 cells without EGCG treatment, the methylation level of *PLAGL1* promoter was significantly reduced after EGCG treatment for 48 h (Figure 1), especially treatment with 150 μ g/mL EGCG resulted in a notable decrease in *PLAGL1* promoter methylation.

Table. 1 Primers Used for Gene Expression Analysis (RT-qPCR).

Primer	Sequence	Annealing temp (°C)
<i>DNMT1-F</i>	5'-CGCTCATTGGCTTTTCTACCG-3'	56
<i>DNMT1-R</i>	5'-AGAACTCGACCACAAATCTT-3'	56
<i>PLAGL1-F</i>	5'-CGCGGCTATCGGGACTG-3'	60
<i>PLAGL1-R</i>	5'-GCCCCGGTTGTCACTACTTA-3'	60
<i>Wnt3a-F</i>	5'-CAAGTGTGGTGCTAAGA-3'	58
<i>Wnt3a-R</i>	5'-AGCAGTTCTTTGTCCGCTGT-3'	58
<i>β-catenin-F</i>	5'-GCGTCTGGTGAAGCATTGTA-3'	59
<i>β-catenin-R</i>	5'-GCGGTGGGGTAAATCAGTAA-3'	59
<i>β-actin-F</i>	5'-GCGTCTGGTGAAGCATTGTA-3'	56
<i>β-actin-R</i>	5'-GCGGTGGGGTAAATCAGTAA-3'	56

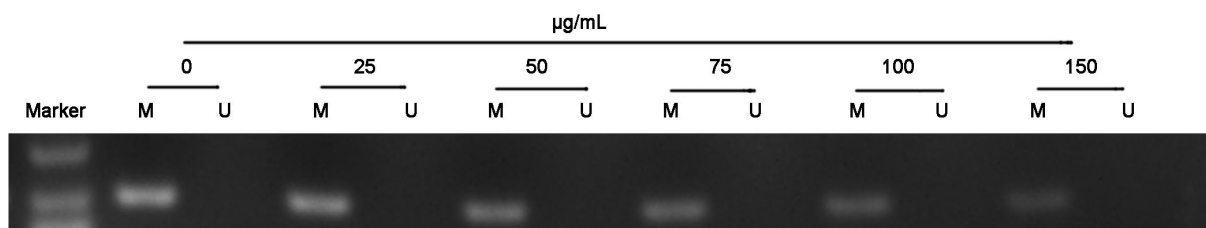


Figure 1. *PLAGL1* Gene Demethylation Induced by EGCG in PC12 Cells. M, Methylation-specific PCR; U, Unmethylation-specific PCR.

Effect of EGCG on the Viability of PC12 Cells

The effects of EGCG on PC12 cell viability was investigated using an MTT assay. The graded concentration of EGCG was used to treat PC12 cells for 48 h, and subsequently the cell viability was measured. As shown in Figure 2, EGCG treatment significantly inhibited the cell viability of PC12 cells in a dose-dependent manner. Treatment with 75 and 100 µg/mL EGCG resulted in 22.79% and 35.11% decrease in the cell viability, whereas treatment with 150 µg/mL EGCG resulted in even greater decrease by 46.38% in PC12 cell line. Compared to PC12 cells without EGCG treatment, the decreases in viability of PC12 cells treated with EGCG were statistically significant ($P<0.05$).

EGCG induces the apoptosis of PC12 cells

The apoptosis of PC12 cells was evaluated using flow cytometry. The PC12 cells were treated using EGCG and then stained with Annexin V and PI. The apoptosis was notably increased by EGCG treatment compared to PC12 cells without EGCG treatment (Figure 3). In addition, treatment with 100 and 150 µg/mL EGCG resulted in even a greater increase by 32.0% and 40.3% respectively. The EGCG induced apoptosis in PC12 cells.

EGCG effect on gene expression levels of DNMT1, PLAGL1, Wnt, and β-catenin

The mRNA levels of *DNMT1*, *PLAGL1*, *Wnt*, and *β-catenin* were measured using RT-qPCR. As shown in Figure 4, treatment with EGCG in PC12 cells significantly

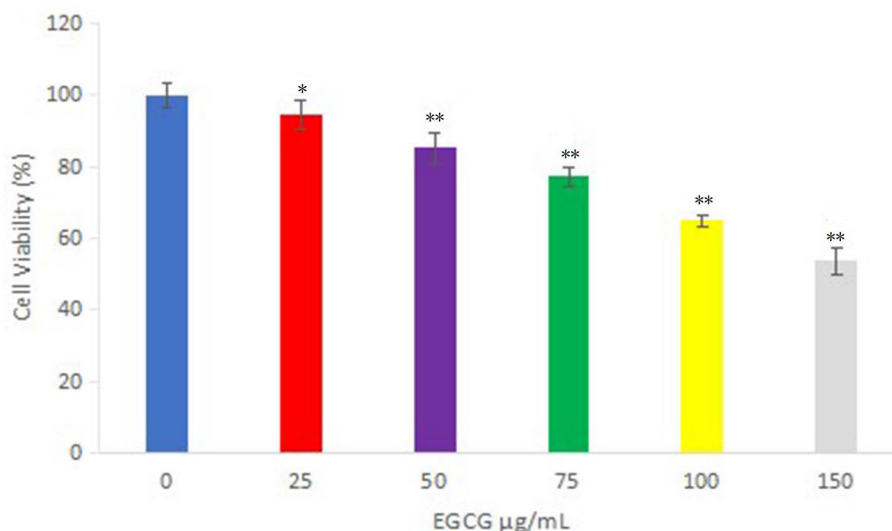


Figure 2. The Effects of EGCG Treatments on Cell Viability Compared with Control * $P<0.05$, ** $P<0.01$

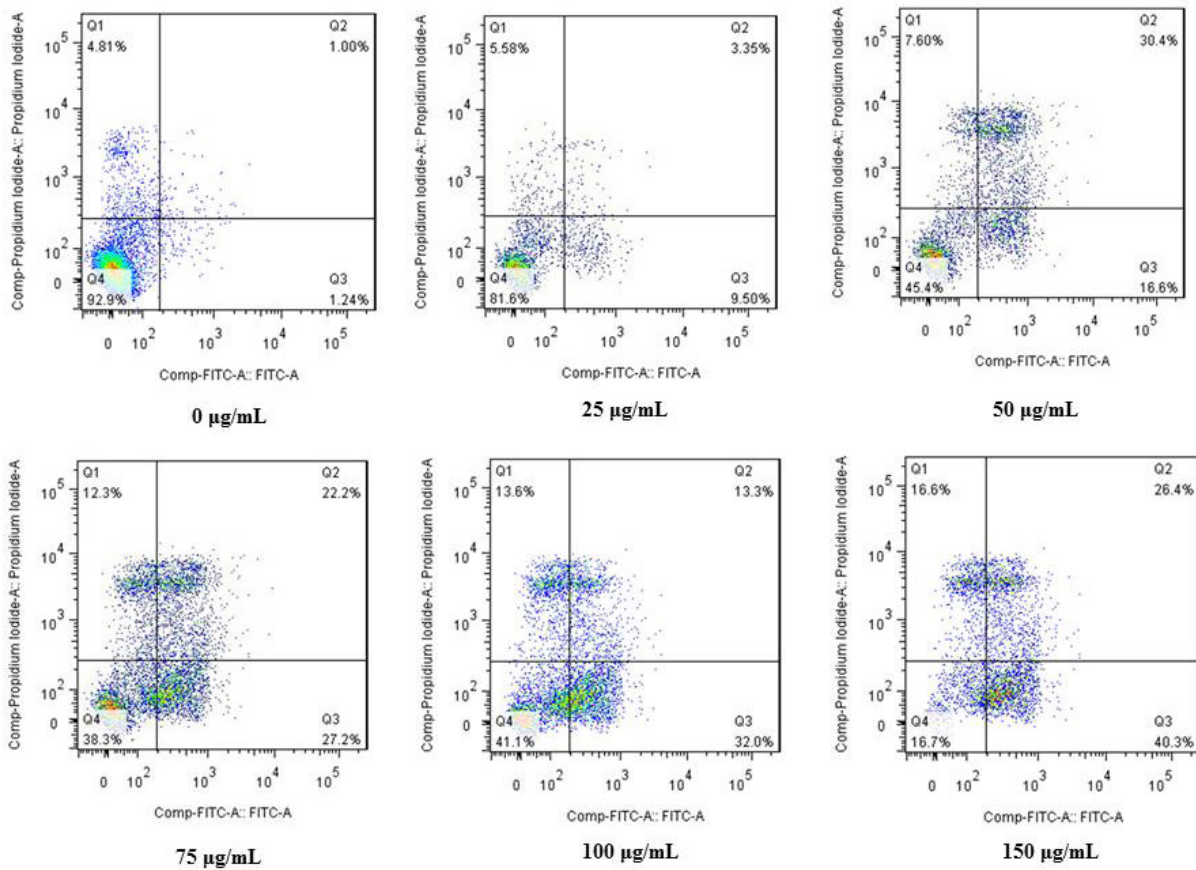


Figure 3. The Effects of EGCG and DDP Treatments on the Cell Apoptosis

increased *PLAGL1* gene expression compared to cells without EGCG treatment, whereas the mRNA levels of *DNMT1*, *Wnt*, and *β-catenin* genes were significantly down-regulated by treatment by EGCG.

EGCG effect on protein expression levels of DNMT1, PLAGL1, Wnt, and β-catenin

The protein expression changes of DNMT1, PLAGL1, Wnt, and β-catenin in PC12 cells were analyzed by western blot. After PC12 cells treatment with EGCG, the

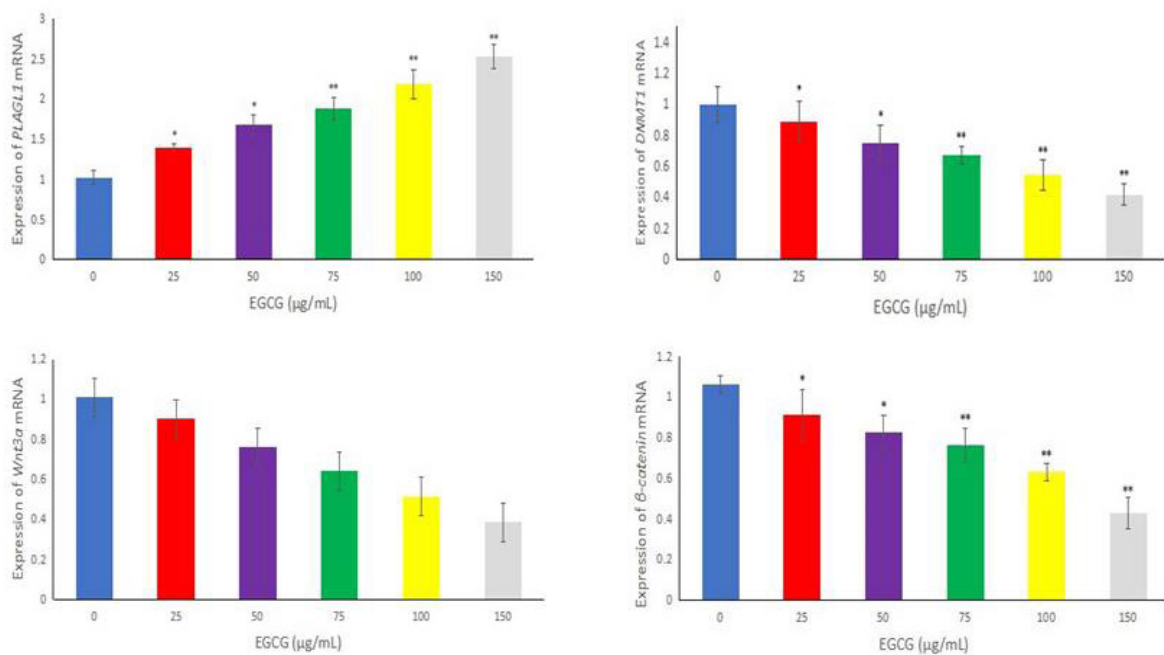


Figure 4. *PLAGL1*, *DNMT1*, *Wnt3a* and *β-catenin* Gene Expressions were Evaluated by RT-qPCR Compared with control * $P < 0.05$, ** $P < 0.01$

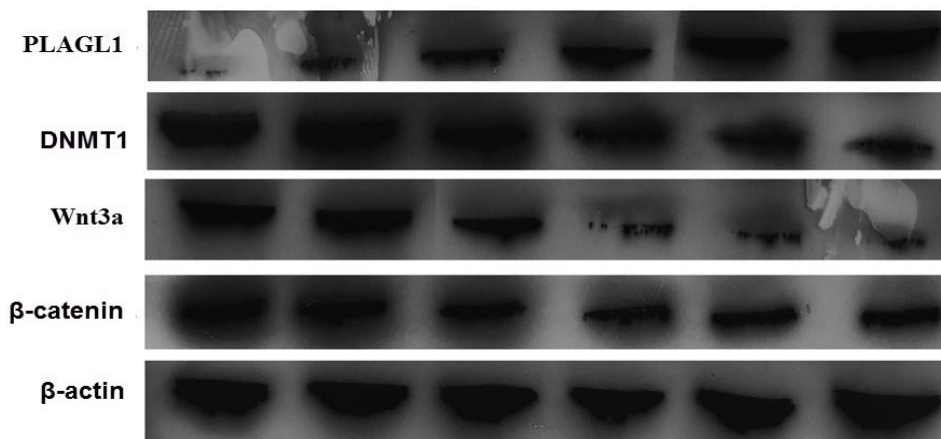


Figure 5. Western Blot was Used to Detect PLAGL1, DNMT1, Wnt3a and β -catenin Protein Expressions Compared with Control * $P < 0.05$, ** $P < 0.01$

protein expression was changed (Figure 5). The PLAGL1 protein levels increased in PC12 cells after treatment with EGCG, whereas the protein expression levels of DNMT1, Wnt, and β -catenin were significantly down-regulated after treatment with EGCG compared to cells without EGCG treatment.

Discussion

Extensive studies have suggested chromosome abnormalities, oncogene mutation or amplification activation, and tumor suppressor gene inactivation play a very important role in the pathogenesis and progress of malignant tumors (Wang et al., 2018; Foggetti et al., 2021; Masclef et al., 2021; Alqahtani et al., 2019). PCC was rare neuroendocrine tumors that arise from chromaffin cells, the prognosis of malignant PCC was poor, and the average 5-year survival rate was 40% (Hamidi, 2019). Although several hereditary syndromes are known to be associated with PCCs, studies have shown some genetic abnormalities including the tumor suppressor gene. Inactivation was involved in the tumorigenesis (Yokota et al., 1991). Brewer (2006) revealed p16 inactivation resulted in the pathogenesis of PCC.

Genetic events including DNA methylation, histone methylation, and deacetylation play a crucial role in the inactivation of tumor suppressor genes in tumors. In human cancers, DNA methylation of tumor suppressor genes is one of the most important epigenetic hallmarks (Hanahan and Weinberg, 2011). Accumulating data demonstrate aberrant hypermethylation of tumor suppressor genes was associated with the development and progress of PCC. Wang analyzed the methylation status of CpG islands in ARHI promoter and aberrant hypermethylation of ARHI was found in PCC compared with normal tissue, indicating ARHI is a novel epigenetic silenced tumor suppressor gene and involved in tumorigenesis of PCC (Wang et al., 2017). Recent studies have uncovered new epigenetic events in PCC and have shown that VHL is epigenetically inactivated in PCC (Andreasson et al., 2013). Recently, reversing the hypermethylation of tumor suppressor genes in cancer for therapeutic purposes have gained traction (Morris et al., 2016). However, some

epigenetically targeted agents such as 5-azacytidine have multiple side effects and efficient epigenetic regulators with low cytotoxicity for the treatment of esophageal cancer are required (Hu et al., 2021). Numerous studies have shown treatment with EGCG in cancer cells affected the methylation status of tumor suppressor genes (Sheng et al., 2019). In this study, we observed the effect of EGCG on PLAGL1 promoter methylation in PC12 cells and investigated the underlying epigenetic mechanism.

PLAGL1 is a tumor suppressor gene and demonstrated to be inactivated in numerous human cancers. Jarmalaite (2011) reported frequent PLAGL1 promoter hypermethylation resulted in inactivation of PLAGL1 expression in PCC. Analysis of DNA methylation in PLAGL1 promoter revealed higher methylation rate in tumor tissues from PCC patients compared to the normal adrenal medulla tissues. In addition, the loss of the active PLAGL1 existed in 65% of PCC patients. Using an MSP assay in PC12 cells, this study revealed that EGCG inhibited promoter methylation of PLAGL1 in a dose-dependent way. The RT-PCR results showed that the expression of PLAGL1 mRNA in PC12 cells was down-regulated and EGCG increased the expression of PLAGL1 mRNA and inhibited the expression of DNMT, indicating EGCG restored the expression of silenced PLAGL1 gene through reducing the activity of DNMT and reversing the hypermethylation of PLAGL1 gene.

In addition to down-regulating DNMT activity by EGCG, EGCG played a role in inducing cell apoptosis, inhibiting epidermal growth factor receptor mediated signal pathway, and reducing oxidative stress (Yokoyama et al., 2004; Wang et al., 2018; Crous-Maso et al., 2018). In this manuscript, the MTT method was used to confirm the effect of EGCG on PC12 cells, the cell viability was significantly suppressed after EGCG treatment, and treatment with 150 μ M EGCG showed maternal inhibition. Similar to our results, Huang (2017) confirmed the inhibitory effect of EGCG on the proliferation of breast cancer MCF-7 cells. Flow cytometry was performed to further elucidate the cell apoptosis by EGCG treatment. The results of the annexin V/PI staining demonstrated that EGCG induced apoptosis in PC12 cells. Thus, EGCG treatment severely affects proliferation of PC12 cells, due

to the induction of cell apoptosis.

The published data showed PLAGL1 suppressed cell proliferation via regulating Wnt/ β -catenin signaling pathway (Zhu et al., 2009). To elucidate the potential molecular mechanism behind EGCG regulating methylation of *PLAGL1* and promoting apoptosis we explored whether Wnt/ β -catenin signaling pathway was associated with the effect of EGCG inducing apoptosis on PC12 cells. The EGCG significantly down-regulated the mRNA and protein expression levels of Wnt and β -catenin, suggesting that demonstrated EGCG contributes to the cell apoptosis in PC12 cells through demethylation of *PLAGL1* involving Wnt/ β -catenin signaling pathway. Thus, it is further confirmed that EGCG can be used as a potential targeted epigenetic drug for PCC.

In conclusion, EGCG can reverse DNA methylation of PC12 cells by reducing the expression of DNMT along with restoring the expression of *PLAGL1* gene, and promoting cell apoptosis via regulating Wnt/ β -catenin signaling pathway. The EGCG can be used as an epigenetic modifier targeting the functional gene, which provides a reference for the clinical application of EGCG in the treatment of PCC. However, the underlying molecular mechanism of EGCG as an Epigenetic drug in vivo needs to be further clarified.

Author Contribution Statement

M.W. and T. D. conceived and designed the research. C. H., J. Z. and D. W. performed the experiments. R. W. and J. Z. analyzed the data. T. D. and C. H. wrote the manuscript.

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Ethical Statement

No animal and human specimens were used in the experiments, our study did not require an ethical board approval.

Data Availability Statement

Data are provided within the article.

Conflict of interest

No potential conflict of interest was reported by the authors.

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- via reducing SCUBE2 methylation and DNMT activity. *Molecules*, **24**, E2899.
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