

A genome-wide study of DNA methylation modified by epigallocatechin-3-gallate in the CAL-27 cell line

LI-LI CHEN^{1,2}, WEN-FEI HAN¹, YING GENG¹ and JIAN-SHENG SU¹

¹Laboratory of Oral Biomedical Science and Translational Medicine, School of Stomatology, Tongji University, Shanghai 200072;

²Department of Stomatology, Zhongshan Hospital, Xiamen University, Xiamen, Fujian 361004, P.R. China

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Abstract. In order to gain greater understanding of the mechanisms underlying the effect of epigallocatechin-3-gallate (EGCG) on DNA methylation and its chemopreventative action in oral squamous cell carcinoma (OSCC), a genome-wide methylation and mRNA expression screen was performed in the CAL-27 cell line with and without EGCG (100 μ M) treatment. A total of 761 differentially methylated gene loci were identified following treatment with EGCG. Comparison of gene expression profiling in OSCC samples revealed 184 transcripts with a significant difference ($P < 0.05$) and a fold change difference > 2 compared with controls. Gene ontology analysis of differentially methylated loci and functional annotation of the differentially expressed genes indicated that the main pathways involved were metabolic, mitogen-activated protein kinase (MAPK), wnt, and cell cycle pathways. In conclusion, the present study indicates that EGCG can affect the methylation status and gene expression in the CAL-27 cell line. Additionally, the changes in several important signaling pathways may reveal the antitumor mechanism of EGCG.

Introduction

Green tea is one of the most popular beverages consumed worldwide, and its tumor-suppressive effects have been reported in various types of cancer, including human head and neck squamous cell carcinoma (1). Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, is considered to have the predominant anticancer and cancer-preventive effects (2).

EGCG inhibits carcinogen activity, tumorigenesis, proliferation and angiogenesis, and induces cell death. These effects are associated with modulation of reactive oxygen species (ROS) production and nuclear factor- κ B, mitogen-activated

protein kinase (MAPK), epidermal growth factor receptor and insulin-like growth factor-I signaling pathways, affecting diverse processes, such as proliferation, differentiation, apoptosis, angiogenesis, metastasis and migration (3). In addition, EGCG could also induce epigenetic modification by inhibition of DNA methyltransferase activity and regulation of histone acetylation, leading to an upregulation of apoptosis (4).

Alterations in DNA methylation, including hypomethylation of oncogenes and hypermethylation of tumor suppressor genes, particularly the hypermethylation of promoter CpG islands are critical in cancer progression (5). Oral squamous cell carcinoma (OSCC) represents the eighth most common type of malignancy in males and the 13th most common type in females worldwide, accounting for ~38% of all head and neck tumors (6). Previous studies have demonstrated that EGCG has cancer-preventative activity in OSCC development (7-9). Mechanistically, this may occur via epigenetic modulation. To date, several studies have investigated DNA methylation and EGCG (10,11). These have predominantly focused on the ability of EGCG to inhibit DNA methyltransferase (DNMT), which leads to the reactivation of genes silenced by promoter or enhancer methylation, including p16^{INK4a}, RAR β , MGMT, hMIH1, WIF-1 and hTERT (12,13). Although these studies were not specific to the development of OSCC, they have indicated that EGCG is capable of affecting genes involved in carcinogenesis, via an epigenetic pathway.

To better understand the mechanisms underlying the effect of EGCG on DNA methylation and its chemopreventative action in OSCC, DNA methylation and mRNA expression profiling in CAL-27 cells treated with EGCG was analyzed.

Materials and methods

Cell culture and drug treatment. The CAL-27 human OSCC cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, GE Healthcare, Little Chalfont, UK) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, GE Healthcare), penicillin (100 IU ml⁻¹) and streptomycin (100 μ g ml⁻¹), and maintained at 37°C in a 5% CO₂ atmosphere. EGCG (14) was obtained from Sigma Aldrich (Carlsbad, CA, USA) (E4143 EGCG, $\geq 95\%$) and freshly prepared each time prior to use. For

Correspondence to: Dr Jian-Sheng Su, Laboratory of Oral Biomedical Science and Translational Medicine, School of Stomatology, Tongji University, 399 Yanchang Middle Road, Shanghai 200072, P.R. China
E-mail: jierduncell@126.com

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microarray analysis, cells were left untreated or were treated with 100 μ M EGCG for 24 h, then genomic DNA and RNA were isolated.

Cell proliferation assay. The effect of EGCG on CAL-27 cell growth was assessed by an MTT cell proliferation and cytotoxicity detection kit (KeyGen Biotech, Nanjing, China). Cells (7×10^4) were seeded onto 96-well plates and grown to 80% confluence prior to treatment with the indicated concentrations of EGCG (0–200 μ M). After 24 h, cell viability was determined by measuring absorbance at 490 nm using a microplate reader (Bio-Rad, Richmond, CA, USA). All assays were performed in triplicate.

Genome-wide methylation assay and statistical analysis. The Infinium II Methylation assay (Illumina, San Diego, CA, USA) was used to detect 27,578 CpG sites genome-wide, spanning 14,495 genes. Illumina chip technology quantifies methylation levels at specific loci within the genome. Following bisulfite treatment, the unmethylated cytosine is converted into uracil and methylated cytosine remains unchanged. Following hybridization with the methylation-specific probe or the non-methylation probe, single-base extension with labelled dideoxynucleotides is performed. Finally, the fluorescence intensities of the methylated and unmethylated signals are measured at each targeted cytosine position to estimate DNA methylation level. Genomic DNA was extracted using the Genomic DNA Mini Preparation kit with Spin Columns (Beyotime Biotech., Jiangsu, China). Bisulfite conversion of genomic DNA from EGCG-treated ($n=3$) and control ($n=3$) samples, was performed using the Zymo EZ DNA methylation kit (Illumina). Samples were run using the automated process on the Infinium methylation BeadChips, in accordance with the manufacturer's instructions. Data were analyzed using BeadStudio Gene Expression Module v3.4 (Illumina).

All differential methylation analysis algorithms compared a group of samples with EGCG treatment to a control group. This comparison was made using the following error models: Illumina Custom Model. P-values were calculated using the following formula:

$$p = z \left(\frac{|\beta_{\text{cond}} - \beta_{\text{ref}}|}{\sqrt{\frac{s_{\text{ref}}^2}{N_{\text{ref}}} + \frac{s_{\text{cond}}^2}{N_{\text{cond}}}}} \right) \quad \beta = \left(\frac{\text{Max}(\text{Cy}5,0)}{\text{Max}(\text{Cy}3,0) + \text{Max}(\text{Cy}5,0) + 100} \right)$$

Where z is the two-sided tail probability of the standard normal distribution; S_{ref} and S_{cond} are standard deviations of probe signals; and N_{ref} and N_{cond} denote the number of samples in the reference (treatment without EGCG) and condition (treatment with EGCG) groups, respectively. β =methylated signal/(unmethylated signal+methylated signal+100). To identify possible cellular functions of these genes, the Gene Ontology analysis tool AmiGO was used (amigo.geneontology.org/cgi-bin/amigo/browse.cgi) (15).

Gene expression and statistical analysis. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The concentration of RNA was determined by NanoDrop 8000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). For amplification and labeling of the RNA with

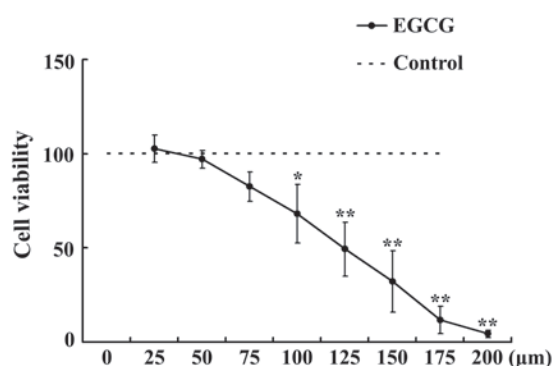


Figure 1. Effect of various doses of EGCG on CAL-27 cell proliferation after 24 h exposure. * $P<0.05$, ** $P<0.01$ vs. control.

the Illumina TotalPrep RNA Amplification kit, 200 ng RNA from each sample was used. The Illumina Sentrix arrays were processed according to the manufacturer's instructions. Slides were scanned immediately using Illumina BeadStation iScan. Data were analyzed using GenomeStudio Gene Expression Module v1.0 (Illumina).

All differential expression algorithms compared a group of samples with EGCG treatment with a control group. The comparison was performed using unpaired Welch's t-test for unequal variance. Genes of which expression was significantly different between the compared groups were selected based on a fold change >2 in gene expression as determined by a t-test, and a $P<0.05$ and corrected for multiple testing using the Benjamin-Hochberg method.

Results

Effect of EGCG on CAL-27 cell proliferation. The effect of various doses of EGCG on CAL-27 cell proliferation was investigated after 24 h exposure. As shown in Fig. 1, EGCG treatment significantly inhibited cell growth in a concentration-dependent manner. Inhibition of proliferation was clearly observed at a concentration of 100 μ M EGCG, and this concentration was therefore utilized in methylation profiling experiments.

Genome-wide differential DNA methylation profiling in EGCG-treated cells. To identify loci that were differentially methylated in CAL-27 cells in response to treatment with EGCG, the Infinium II Methylation assay was used, which interrogated 27,578 loci, covering $>14,000$ genes. Comparison of methylation profiles between EGCG-treated and control samples identified 677 genes ($P<0.05$) and 84 genes ($P<0.01$) that were differentially methylated. Of the 84 genes altered in response to EGCG treatment, 57 were hypermethylated and 24 were hypomethylated (Fig. 2A and B). To identify possible cellular functions of these genes, gene ontology (GO) analysis was performed. Biological functions of hypermethylated genes include transport, cell cycle, transduction, oxidative processes and apoptosis, whereas hypomethylated gene loci were enriched for genes involved in apoptosis, transduction, oxidative processes and cell adhesion (Fig. 2A and B).

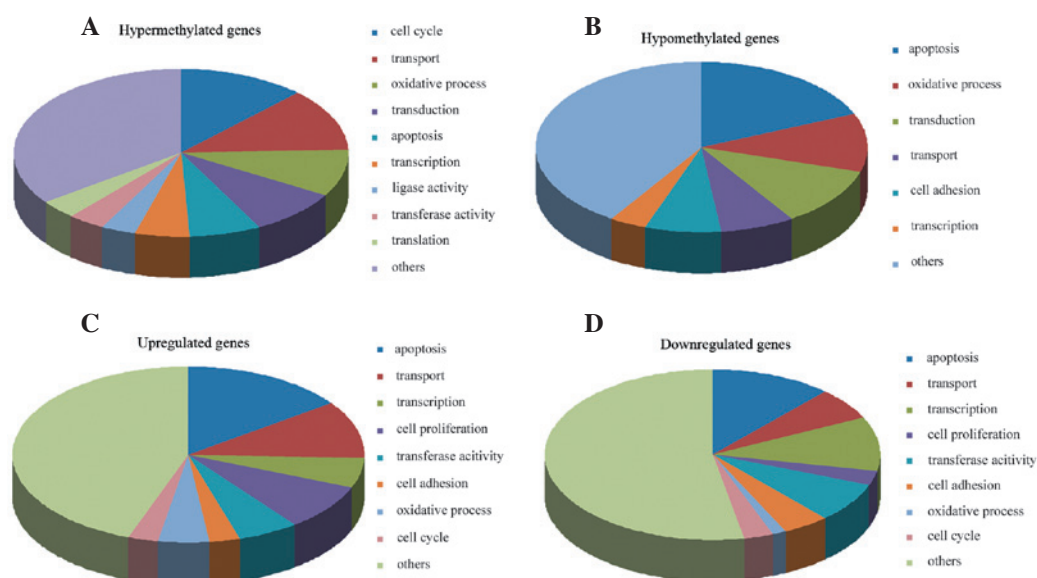


Figure 2. GO analysis (Biological Process) of different genes between EGCG-treated and control CAL-27 cell lines. (A) hypermethylated genes; (B) hypomethylated genes; (C) upregulated genes; (D) downregulated genes.

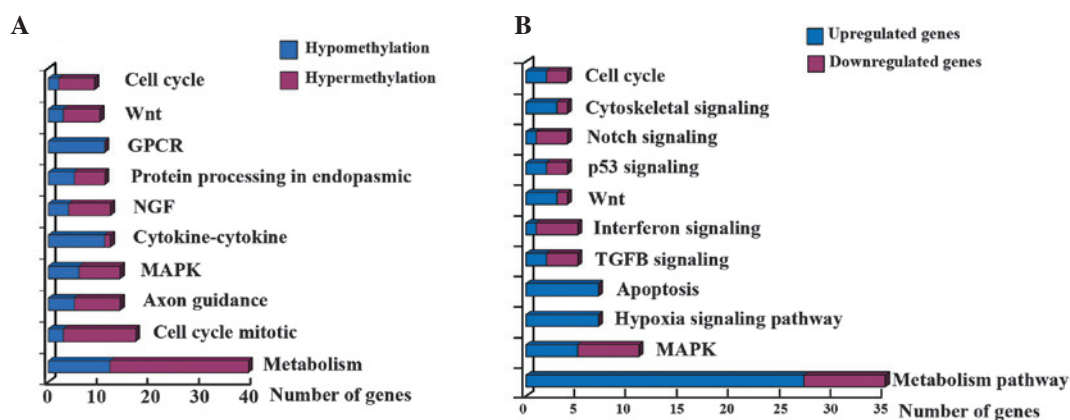


Figure 3. Pathway analysis of different genes between EGCG-treated and control CAL-27 cell lines. (A) Hypermethylated/hypomethylated genes and (B) upregulated/downregulated genes.

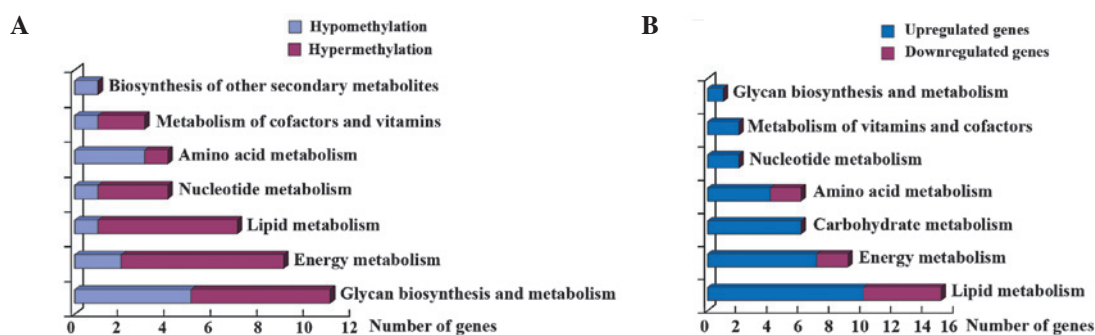


Figure 4. Metabolic pathway analysis of different genes between EGCG-treated and control CAL-27 cell line. (A) Hypermethylated/hypomethylated genes and (B) upregulated/downregulated genes.

Differentially methylated genes are enhanced for specific signaling pathways. To identify signaling pathways associated with EGCG treatment, pathway analyses were performed. Of the 677 genes ($P < 0.05$) differentially methylated following EGCG treatment, 229 genes were associated with signaling

pathways. The top 10 enriched pathways were: Metabolic pathways, cell cycle/mitotic, axon guidance, MAPK signaling, cytokine-cytokine receptor interaction, signaling by nerve growth factor, protein processing in endoplasmic reticulum, signaling by G-protein-coupled receptor, Wnt signaling

pathway and cell cycle (Fig. 3A). Analysis of metabolic pathway genes affected by EGCG treatment identified an enrichment of genes involved in glycan biosynthesis and metabolism, energy metabolism and lipid metabolism (Fig. 4A). The other 84 genes ($P < 0.01$) were also analyzed but no enriched pathways were identified.

Differential gene RNA expression. Whole-genome gene expression analysis was performed on RNA samples isolated from CAL-27 cells with and without EGCG treatment. One hundred and eighty-four transcripts showed a fold change difference >2 in gene expression after EGCG treatment in CAL-27 cells from 47,317 detected probes in the array. One hundred and fifteen transcripts were upregulated and 69 downregulated among the 184 transcripts that were significantly changed after EGCG treatment. Up- and downregulated genes were involved in the following biological functions: Apoptosis, transport, transcription, cell proliferation, transferase activity, cell adhesion, oxidative processes and the cell cycle (Fig. 2C and D).

Pathway analyses indicated that the main pathways associated with the differentially expressed genes were: Metabolism pathway, MAPK, hypoxia signaling pathway, apoptosis, transforming growth factor β signaling, interferon signaling, Wnt, p53 signaling, Notch signaling, cytoskeletal signaling and cell cycle (Fig. 3B).

Analysis of metabolic pathway genes affected by EGCG treatment identified an enrichment of genes involved in lipid, energy and carbohydrate metabolism (Fig. 4B).

Discussion

A number of studies using cell culture and animal models have suggested that EGCG is a prospective candidate for use in the chemoprevention of cancer (16-22). Anticancer effects of EGCG include: Inhibition of carcinogen activity and tumorigenesis; inhibition of tumor proliferation and angiogenesis; inhibition of tumor migration and invasion; and induction of cell death (23). These effects have also been found in OSCC, including in the CAL-27 cell line (24-26). However, to the best of our knowledge, the mechanisms underlying EGCG action have not been clarified. DNA methylation is the most extensively investigated epigenetic modification. Hypermethylation on the DNA molecule limits the binding of transcription factors to promoters, resulting in the recruitment of additional proteins and gene silencing (27). This methylation is mediated by DNMT. EGCG is known to be an inhibitor of DNMT by direct inhibitory interaction with the catalytic site of DNMT (28). Several studies have found that EGCG reverses the methylation-mediated downregulation of the tumor suppressors and then reduces cell growth and colony formation (29). However, few studies have investigated the effect of EGCG on global methylation. The demethylation effect of EGCG on the matrix metalloproteinase inhibitor, RECK, is the only report of EGCG modulating DNA methylation in oral carcinoma cells (30).

In the present study, EGCG treatment significantly inhibited the proliferation of CAL-27 cells after 24 h, in a concentration-dependent manner. These results are consistent with our previous findings that, at 100 μ M EGCG, the survival

rate of CAL-27 cells rapidly declined; however, the survival rate of human gingival fibroblasts was not affected, even after 100 μ M EGCG treatment for 72 h. Hence, 100 μ M EGCG was used to treat CAL-27 cells, which does not harm normal oral cells but inhibits the growth of oral cancer cells.

This study represents the first genome-wide methylation analysis of genes affected by EGCG. EGCG treatment of CAL-27 cells leads to hypermethylation and hypomethylation of gene loci, while EGCG treatment was associated with higher levels of hypermethylation compared with hypomethylation (57 vs. 27 genes). Whole genome expression analysis indicated that the expression of 184 transcripts was altered. Although the majority of genes with altered methylation showed no significant changes in expression, the function and pathway analyses indicated the anti-tumor effects of EGCG. Analysis of genes with changed methylation status revealed their functions are involved in the regulation of the cell cycle, transport, oxidative processes, apoptosis, transcription, transferase activity and cell adhesion. These functions are consistent with the analysis of genes with changed expression levels and strongly indicate the anticancer properties of EGCG.

Integrated analysis of DNA methylation and mRNA expression showed that four pathways were significantly changed by EGCG treatment: Metabolism, MAPK, Wnt and cell cycle pathways. A large portion of genes that were altered upon treatment with EGCG were involved in metabolism pathways. The ability of EGCG to affect metabolism may explain why it is considered to be beneficial in the prevention and/or treatment of cardiovascular and metabolic diseases, such as obesity and diabetes mellitus (31). Given that metabolism pathways are essential for cancer cells, they may be an important target from EGCG, contributing to its inhibitory effect on tumorigenesis.

MAPKs are composed of extracellular signal-regulated kinase, p38 MAPK, and c-Jun N-terminal kinase, and the deregulation of MAPK cascades contributes to cancer. MAPK signaling has previously been identified as a target for cancer prevention by EGCG (32), the present data show that it may be regulated via an epigenetic mechanism.

Aberrant regulation of the Wnt signaling pathway exhibits an important role in cancer biology. Kim *et al* (33) found that EGCG inhibits Wnt signaling and the Wnt target gene c-MYC in breast cancer cells by inducing the HBP1 transcriptional repressor and inhibiting aspects of invasive breast cancer. According to the data of the present study, EGCG changed the methylation status of CSNK1E, CSNK2A1, LRP6, MYC, NFATC4, SMSD4, TCF7 and TCF7L1, and altered gene expression of ANGPTL4, DAB2, NDRG1 and CXXC5, which was associated with the Wnt signaling pathway.

The cell cycle is a series of events that takes place in a cell leading to its division and duplication. Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. EGCG downregulated the cell cycle regulatory proteins CCND1 and PPP2R2B, and upregulated CDKN1C. This may have occurred via epigenetic mechanisms. Other pathways, such as apoptosis and p53 signaling, are classical antitumor pathways, their dysregulation following EGCG treatment strongly indicated the antitumorigenic activity of EGCG.

In conclusion, to the best of our knowledge, the present study reported for the first time the genome-wide analysis of promoter methylation and expression profiling in the OSCC cell line treated with EGCG. Additionally, the changes in several important signaling pathways may reveal the antitumor mechanism of EGCG.

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