Abstract. Background: Aberrant promoter methylation of Wnt inhibitory factor-1 (WIF-1) is a fundamental mechanism of epigenetic silencing in human cancers. Epigallocatechin-3-gallate (EGCG) has been reported to directly reactivate several methylation-silenced genes. The promoter demethylation and reactivation of WIF-1 has not previously been reported. Materials and Methods: Methylation-specific PCR, sequencing analysis and RT-PCR analysis were performed to evaluate promoter demethylation of WIF-1 and WIF-1 expression. Western blot analysis and luciferase reporter assay were performed to evaluate expression of cytosolic β-catenin protein and Tcf/Lef reporter activity. Results: Promoter demethylation of WIF-1 and restoration of WIF-1 expression after EGCG treatment are demonstrated in H460 and A549 cell lines. EGCG also decreased cytosolic β-catenin protein level and inhibited Tcf/Lef reporter activity. Conclusion: These results suggest the potential therapeutic use of EGCG for the reversal of WIF-1 promoter methylation.
supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL). All cells were cultured at 37°C in a humid incubator with 5% CO₂. Normal lung tissues from patients undergoing resection for lung cancers were collected at the time of surgery and immediately snap-frozen in liquid nitrogen (IRB# H8714 15319-040). These tissue samples were kept at −170°C in a liquid nitrogen freezer before use. EGCG and 5-Aza-2’-deoxycytidine (DAC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). They were prepared as stock solutions in dimethyl sulfoxide (DMSO), diluted in media and sterile filtered before use. To restore WIF-1 expression, 1x10⁵ cells were seeded into 6-well culture plates. After 24 hours culture period, cells were treated with EGCG (0-50 μM) and DAC (20 μM) for 72 hours, as described previously (25).

Cell proliferation. H460, A549 and HCT116 cells were plated in 96-well plates at a density of 5000 cells/well. Cells were allowed to attach overnight in culture medium. H460 A549 and HCT116 cells were treated with EGCG (0-200 μM). After incubation for 72 hours, cellular proliferation was measured using the MTS assay and absorbance was measured at 490 nm. Data were presented as means±SD.

Semi-quantitative reverse transcription-PCR. Total RNA from H460, A549, HCT116 and H1703 cell lines, and normal lung tissue was isolated using an RNeasy MINI extraction kit (Qiagen, Valencia, CA, USA). Reverse transcription-PCR (RT-PCR) was performed in a Bio-RAD iCycler (Bio-RAD Laboratories, Hercules, CA, USA) using a RT-PCR kit (SuperScript One-step RT-PCR with Platinum Taq kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Primer sequences for the human WIF-1 cDNA were 5’-CCGAAATGGAGGCTTTTGTA-3’ (forward) and 5’-AACGAAACCAACAATCAACG-3’ (reverse). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as an internal control.

Methylation-specific PCR (MSP). Genomic DNA from the cell lines and normal lung tissue was extracted using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. Bisulfite modification of genomic DNA was performed by using a methylation kit (EZDNA methylation kit; Zymo Research, Orange, CA, USA). Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an unmethylation-specific primer set. HotStarTaq DNA polymerase (Qiagen) was used in the experiments. Sequences of the methylation-specific primers were 5’-TCCGGGGGTTTTTATTTGCGGC-3’ (forward) and 5’-AACCAGAAACAAAATCAACG-3’ (reverse). Sequences of the unmethylation-specific primers were 5’- TTGGTTGTTATTTATTGGGTTT-3’ (forward) and 5’-AACAAAAACC AACAATCAACA-3’ (reverse).

Sequencing analysis. Bisulfite-treated genomic DNA was amplified using two pairs of primers: 5’-GATGGATGTGGTTAGGGTTT-3’ (forward) and 5’-CCTAAATACCAAAAAACCTAC-3’ (reverse), designed to amplify nucleotides −554 to −140 of the WIF-1 promoter region; and 5’-GTAGGGTTTGGATATTAGG-3’ (forward) and 5’-TCCATAAATACACCTCCTCCTC-3’ (reverse), to amplify nucleotides −161 to +118 (the start codon ATG of WIF-1 is defined as +1). The PCR products were extracted from the agarose gel using an extraction kit (QIAquick Gel Extraction kit; Qiagen) and were subsequently sequenced at MCLab (South San Francisco, CA, USA).

Transient transfection and luciferase reporter assay. The TOP/FOP flash plasmid system was used to determine the transcriptional activity of TCF. All transfection experiments were performed using the Lipofectamine 2000 method (Invitrogen) in triplicate in accordance with the manufacturer’s instructions. The HCT116 cells were transfected with 8 μg super8xTOP flash or 8 μg super8xFOP flash plasmid (as a kind gift from Professor Randall Moon, Howard Hughes Medical Institute and Department of Pharmacology, University of Washington School of Medicine, Seattle, WA, USA), the pRL-TK plasmid (Promega) was co-transfected to normalize for transfection efficiency. Cells were treated with EGCG (0-50 μM) 24 hours after transfection. Luciferase activity was assayed 24 hours after treatment by using the Dual- Luciferase reporter assay system (Promega, Madison, WI, USA).

Western blot analysis. Cytosolic proteins were prepared as previously described (26). The proteins were separated on 4-15% gradient SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bellerica, MA, USA). The proteins were bound with the primary antibodies of β-catenin (Transduction Laboratories, Lexington, KY, USA) and β-actin (Sigma Chemical, St. Louis, MO, USA). Antigen-antibody complexes were detected by ECL blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis. Data were shown as mean±standard deviation (SD). Multiple comparison with Tukey method was used to compare the mean of TOP/FOP in different concentration groups. Statistical significance was established at p<0.05.

Results

Effect of EGCG on cell proliferation. The effects of EGCG on cell proliferation in H460, A549 and HCT116 cell lines were analyzed using the MTS assay (Figure 1A). It was observed that compared with that of the controls, cell viability was not altered for concentrations up to 50 μM and was reduced for concentrations ranging from 50 to 200 μM in a concentration-dependent manner. Therefore, treatment with EGCG, at concentrations ranging from 0 to 50 μM, has no cytotoxic effects to H460, A549 and HCT116 cells over a 72 h incubation period.

Silencing and promoter methylation of WIF-1 in H460 and A549 cell lines. The WIF-1 transcript was undetectable in H460, A549 and HCT116 cell lines. In contrast, it was expressed in H1703 cells and normal lung tissue (Figure 1B). Promoter methylation was found by using MSP in H460 and A549 cell lines (Figure 2A). Consistent with MSP results, the bisulfite genomic DNA sequencing technique showed that the CpG islands in H460 and A549 cell lines were densely methylated. In contrast, CpG islands in normal lung tissue were in unmethylation status (Figure 2B).

Promoter demethylation of WIF-1 gene with EGCG treatment. The methylation status of CpG islands in the WIF-1 promoter was determined using a bisulfite genomic DNA sequencing technique. The results showed that the CpG islands in H460 and A549 cell lines were densely methylated. In contrast, CpG islands in normal lung tissue were in unmethylation status (Figure 2B).
region in H460 and A549 cell lines was analyzed after incubation with EGCG for 72 h. By using the MSP technique and DAC as a positive demethylation control (15), the WIF-1 promoter region revealed concentration-dependent demethylation (Figure 2A). Bisulfite genomic DNA sequencing was also used to analyze details of the methylation status of 64 CpG sites in the 672 bps of the WIF-1 promoter region. The sequencing results showed that the WIF-1 promoter methylation level decreased from 77.6% to 27.6% after treatment with 20 μM EGCG in the H460 cell line and from 76.5% to 28.6% in the A549 cell line (Figure 2B). These results suggest that WIF-1 silencing in H460 and A549 cell lines correlates with dense methylation of the WIF-1 promoter region and can be restored by EGCG.

**Restoration of WIF-1 expression with EGCG treatment.** The expression level of WIF-1 was analyzed by semi-quantitative RT-PCR. In agreement with the MSP and sequencing data, the results revealed detectable and concentration-dependent reactivation after treatment by EGCG (0-50 μM) for 72 h in the H460 cell line, but no significant concentration-dependent reactivation in the A549 cell line (Figure 3). These results suggest that WIF-1 silencing in H460 and A549 cell lines correlates with dense methylation of the WIF-1 promoter region and can be restored by EGCG.

**Down-regulation of the Wnt canonical pathway.** Down-regulation of the Wnt canonical pathway was confirmed by Tcf/Lef activity assay and cytosolic β-catenin protein level. The HCT116 cells were transfected with TOP/FOP luciferase reporter plasmids. A concentration-dependent decrease of TOP flash luciferase activity with no change in the mutated control (FOP flash) would be expected to correlate with down-
regulation of β-catenin-induced Tcf/Lef transcriptional activity (27) (Figure 4A; *p=0.028). The expression of β-catenin was subsequently measured by Western blot analysis using β-actin as an internal control. Consistent with TOP/FOP results, it was found that H460 and A549 cells treated by EGCG had decreased expression of β-catenin (Figure 4B).
Discussion

The Wnt pathway plays a critical role in oncogenesis and tumor development (28, 29). Several Wnt proteins, including Wnt-1, Dvl-3 and β-catenin, have been shown to be overexpressed in a number of cancers (9-12). WIF-1 inhibits Wnt signaling by direct binding to Wnt molecules, it downregulates the Wnt pathway and inhibits NSCLC cell growth. Transfection with WIF-1 induces apoptosis in NSCLC cells and also inhibits NSCLC tumor xenograft growth (17).

It is well established that aberrant methylation of CpG islands is a key mechanism for inactivating tumor suppressor genes in cancer. A growing list of genes have been identified that show abnormal CpG island promoter methylation (2). In previous studies, it was demonstrated that WIF-1 in lung cancer cell lines is silenced due to promoter hypermethylation (17), and that promoter demethylation of WIF-1 can restore the silenced WIF-1 and down-regulate the canonical Wnt pathway.

EGCG has been extensively studied in the context of its anticancer effects, but its mechanisms of action are not well understood (30-32). It has been reported that EGCG inhibits the DNMT with a Ki of 4.8 μmol/L (33). Molecular modeling of the interaction between EGCG and DNMT revealed a substantial interactive region with hemi-methylated DNA and a cytosine-active pocket for subsequent methylation (24). Further, it has been found to reactivate several methylation-silenced genes such as RARβ and p16 (24,25). It was therefore hypothesized that EGCG can also reactivate the methylation-silenced WIF-1 gene.

The results of this study confirmed that the WIF-1 gene in untreated H460 and A549 cells was silenced by promoter methylation. After EGCG treatment, this epigenetic change was reversed. In addition, we have also shown that H460 and A549 cells treated with EGCG evidenced decreased Tcf/Lef activity and reduced cytosolic β-catenin protein level. These results support our hypothesis that EGCG can, in addition to restoring the methylated WIF-1 by demethylation, down-regulate the Wnt canonical pathway in H460 and A549 cell lines.

Tea is a commonly consumed beverage worldwide. Epidemiological evidence suggests that consuming 1–3 cups or more of green tea daily for greater than a year may reduce the risk of prostate cancer by nearly half (34). EGCG is the major polyphenol of green tea. Our data suggest that green tea acts as a chemopreventive agent partially due to reactivating WIF-1 gene expression and inhibiting the canonical Wnt pathway.

Here, we show the first evidence that EGCG reactivates WIF-1 from a previous silenced state by methylation and also down-regulates the canonical Wnt pathway. This suggests a potential therapeutic use of EGCG for the restoration of WIF-1 expression and reversal of WIF-1 promoter methylation.

Acknowledgements

This work was supported by NIH grant RO1 CA 093708-01A3, the Larry Hall memorial trust and the Kazan, McClain, Edises, Abrams, Fernander, Lyons and Farrise Foundation.

We thank Richard N. Barg, J.D. MBA of the Thoracic Oncology Program and the Department of Surgery at the University of California, San Francisco, for his editorial help in the preparation of this manuscript.

References