Promoter Demethylation of *WIF-1* by Epigallocatechin-3-gallate in Lung Cancer Cells

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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.*

Aberrant promoter methylation of tumor suppressor genes is a fundamental mechanism of epigenetic silencing (1-4). Nearly 50% of all proven tumor suppressor genes can be silenced by hypermethylation along with many genes that play putative roles in antitumor activities (5).

The Wingless-type (Wnt) family consists of a group of signaling molecules that is extensively involved in developmental processes and oncogenesis (6,7). The proto-oncogenic effects of Wnt genes were discovered more than 20 years ago (8). Since then, numerous reports have demonstrated aberrant activation of the Wnt signaling pathway in disparate human cancers such as colorectal cancer (9), head and neck carcinoma (10), melanoma (11) and leukemia (12). The over-expression of disheveled (Dvl) and β-catenin proteins in mesothelioma, nasopharyngeal carcinoma (NPC) and non-small cell lung cancer (NSCLC) has previously been reported (13-15).

Wnt inhibitory factor-1 (WIF-1) is a Wnt antagonist that inhibits Wnt signaling by direct binding to Wnt molecules. In previous studies, the *WIF-1* promoter has been cloned (16), and more importantly, it has been shown that *WIF-1* is silenced due to promoter hypermethylation in NSCLC, NPC, mesothelioma, melanoma and Barrett's esophagus (17-20). Recently, *WIF-1* silencing due to promoter hypermethylation in other malignancies was also reported (18). Re-expression of *WIF-1* can down-regulate the Wnt pathway and inhibit cancer cell growth (17, 18).

Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea. It has been shown that EGCG is methylated by catechol-O-methyltransferase to form MeEGCG and DiMeEGCG both *in vitro* and *in vivo* (21, 22). EGCG is also an inhibitor of catechol-O-methyltransferase (22) and DNA methyltransferase (DNMT) (23, 24). It was reported that EGCG can reactivate several methylation-silenced genes such as RARβ and p16. The promoter demethylation of *WIF-1* has not previously been reported. Our hypothesis is that EGCG can be used as an effective and non-toxic demethylating agent of *WIF-1* promoter.

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Materials and Methods

Cell lines, tissue samples and chemical treatment. NSCLC cell lines (H1703, H460 and A549) and colorectal cancer cell line HCT116 were obtained from American Type Culture Collections (Manassas, VA, USA). Cells were cultured in RPMI 1640

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% CO_2. 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, 1×10^5 cells were seeded into 6-well culture plates. After 24 hours culture, 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'-GTGTCTTCCATGCCAACCTT-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'-TCGCGGGCGTTTTATTGGGC-3' (forward) and 5'-AACGAAACCAACAATCAACG-3' (reverse). Sequences of the unmethylation-specific primers were 5'-TTGTGGGTGTTTTATTGGGT-3' (forward) and 5'-AACAAAACC AACAATCAACA-3' (reverse).

Sequencing analysis. Bisulfite-treated genomic DNA was amplified using two pairs of primers: 5'-GAGTGATGTTTTAGGGGTTT-3' (forward) and 5'-CCTAAATACCAAAAAACCTAC-3' (reverse), designed to amplify nucleotides –554 to –140 of the WIF-1 promoter region; and 5'-GTAGGTTTTTTGGTATTTAGG-3' (forward) and 5'-TCCATAAATACAAACTCTCCTC-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 HCT 116 cells were transfected with 8 μg super8×TOP flash or 8 μg super8×FOP 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 value±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

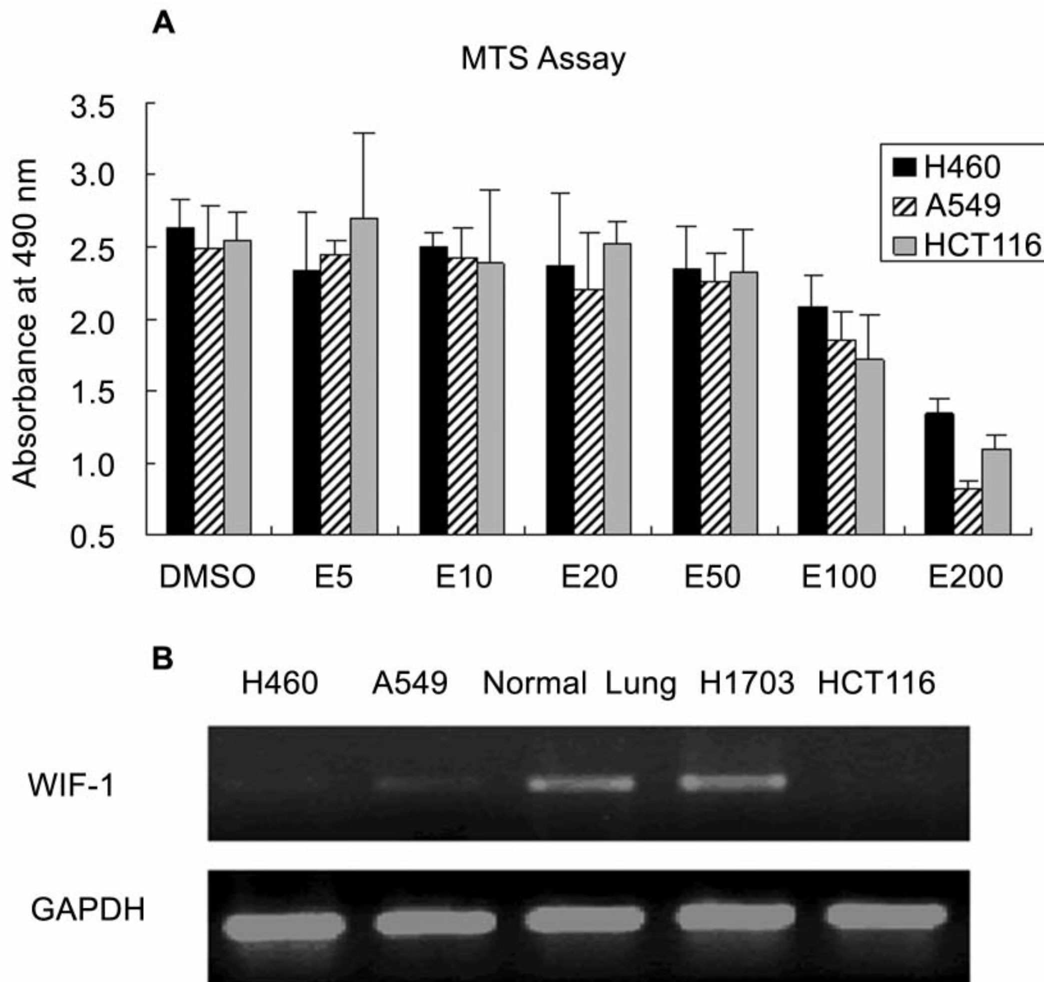

Figure 1. *Growth inhibition of lung cancer cells by EGCG. (A) Proliferation effects of EGCG in H460, A549 and HCT116 cells. Cells were treated with EGCG and incubated for 72 h; proliferation was assessed by MTS assay. Experiments were performed independently at least three times. Control carrier DMSO (0 μg/mL). Data are represented as means±SD. E5-200 represent EGCG concentrations 5-200μM (B) Reverse transcriptase-PCR for WIF-1. The amplified human cDNA fragment is 451 bp. the H1703 and HCT116 were included as positive and negative controls respectively, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for RNA quality and loading.*

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).

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-

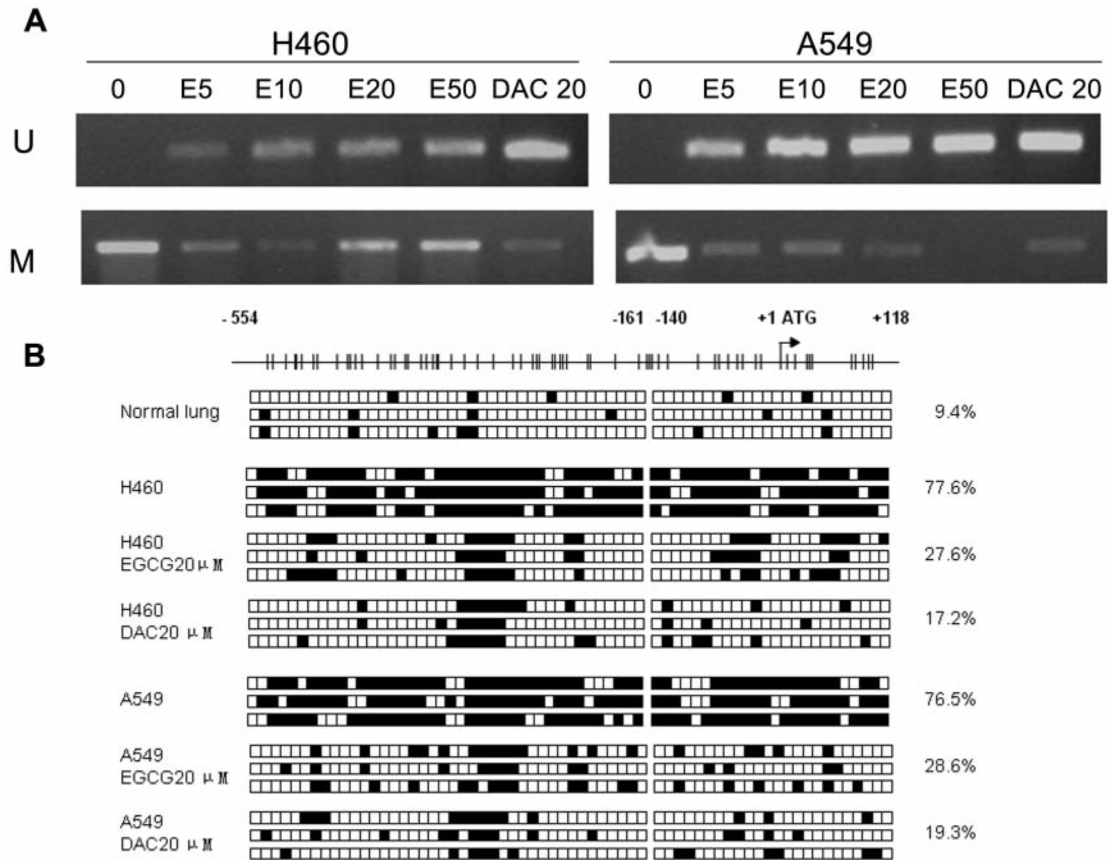

Figure 2. *Methylation and pharmacological demethylation of WIF-1 promotor region with EGCG and DAC treatment in H460 and A549 cells. (A) Methylation-specific PCR analysis of the CpG island of cell lines treated by EGCG (5-50 μM) DAC was used as a positive demethylation control. Bands in Lane U are unmethylated DNA product with unmethylation-specific primers. Bands in Lane M are methylated DNA product amplified with methylation-specific primers. (B) Bisulfite genomic DNA sequencing analysis; □ and ■ represent unmethylated and methylated CpG islands, respectively, percentage indicates the fraction of methylated CpG islands. Three clones of PCR products amplified from bisulfite-treated genomic DNA were sequenced for each cell line.*

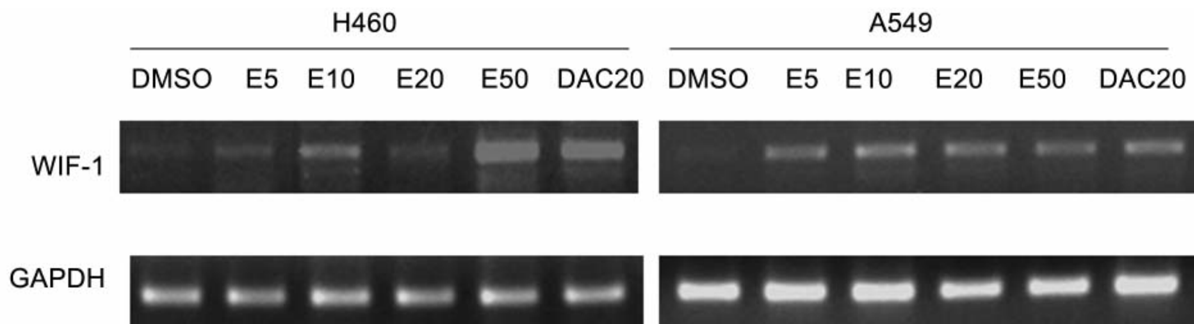

Figure 3. *Reactivation of WIF-1 expression in cell lines after EGCG treatment. Reverse transcription-PCR was performed after EGCG (5-50 μM) and DAC (20 μM) treatment for 72 h.*

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).

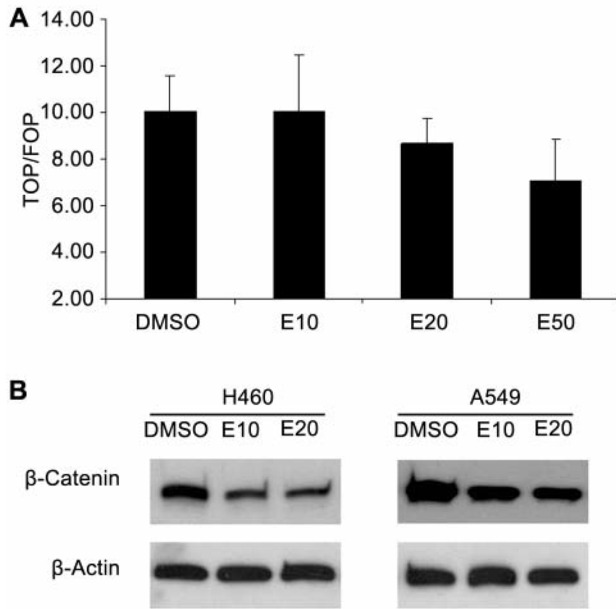

Figure 4. *Inhibition of Wnt canonical pathway by EGCG. (A) TOP/FOP measurement by using the Dual-Luciferase reporter assay system. Cells were treated with EGCG (0-50 μM). Data are represented as means±SD. (B) Expression of cytosolic β-catenin protein after EGCG treatment. H460 and A549 cells were treated with 10 and 20 μM EGCG for 72 h; protein levels were determined with Western blot analysis using β-actin as an internal control.*

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 over-expressed in a number of cancers (9-12). WIF-1 inhibits Wnt signaling by direct binding to Wnt molecules, it down-regulates 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.

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References

1 Antequera F and Bird A: Number of cpg islands and genes in human and mouse. Proc Natl Acad Sci USA *90*: 11995-11999, 1993.

2 Herman JG and Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med *349*: 2042-2054, 2003.

3 Rountree MR, Bachman KE, Herman JG and Baylin SB: DNA methylation, chromatin inheritance, and cancer. Oncogene *20*: 3156-3165, 2001.

4 Jones PA and Laird PW: Cancer epigenetics comes of age. Nat Genet *21*: 163-167, 1999.

5 Baylin SB: Reversal of gene silencing as a therapeutic target for cancer – roles for DNA methylation and its interdigitation with chromatin. Novartis Found Symp *259*: 226-233, 2004; discussion 234-227, 285-228.

6 Lustig B and Behrens J: The wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol *129*: 199-221, 2003.
7 Polakis P: Wnt signaling and cancer. Genes Dev *14*: 1837-1851, 2000.
8 Nusse R and Varmus HE: Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*: 99-109, 1982.
9 Yue W, Sun Q, Dacic S, Landreneau R, Siegfried JM, Yu J and Zhang L: Down-regulation of dkk3 activates -catenin/tcf-4 signaling in lung cancer. Carcinogenesis *29*: 84-92, 2008.
10 Rhee CS, Sen M, Lu D, Wu C, Leoni L, Rubin J, Corr M, Carson DA: Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. Oncogene *21*: 6598-6605, 2002.
11 Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M and Trent JM: Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell *1*: 279-288, 2002.
12 Lu D, Zhao Y, Tawatao R, Cottam HB, Sen M, Leoni LM, Kipps TJ, Corr M and Carson DA: Activation of the wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci USA *101*: 3118-3123, 2004.
13 Lin YC, You L, Xu Z, He B, Yang CT, Chen JK, Mikami I, Clement G, Shi Y, Kuchenbecker K, Okamoto J, Kashani-Sabet M and Jablons DM: Wnt inhibitory factor-1 gene transfer inhibits melanoma cell growth. Hum Gene Ther *18*: 379-386, 2007.
14 Uematsu K, He B, You L, Xu Z, McCormick F and Jablons DM: Activation of the wnt pathway in non small cell lung cancer: Evidence of dishevelled overexpression. Oncogene *22*: 7218-7221, 2003.
15 Lin YC, You L, Xu Z, He B, Mikami I, Thung E, Chou J, Kuchenbecker K, Kim J, Raz D, Yang CT, Chen JK, Jablons DM: Wnt signaling activation and wif-1 silencing in nasopharyngeal cancer cell lines. Biochem Biophys Res Commun 341:635-640, 2006.
16 Reguart N, He B, Xu Z, You L, Lee AY, Mazieres J, Mikami I, Batra S, Rosell R, McCormick F and Jablons DM: Cloning and characterization of the promoter of human wnt inhibitory factor-1. Biochem Biophys Res Commun *323*: 229-234, 2004.
17 Mazieres J, He B, You L, Xu Z, Lee AY, Mikami I, Reguart N, Rosell R, McCormick F and Jablons DM: Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. Cancer Res *64*: 4717-4720, 2004.
18 Taniguchi H, Yamamoto H, Hirata T, Miyamoto N, Oki M, Nosho K, Adachi Y, Endo T, Imai K and Shinomura Y: Frequent epigenetic inactivation of wnt inhibitory factor-1 in human gastrointestinal cancers. Oncogene *24*: 7946-7952, 2005.
19 Yang TM, Leu SW, Li JM, Hung MS, Lin CH, Lin YC, Huang TJ, Tsai YH and Yang CT: Wif-1 promoter region hypermethylation as an adjuvant diagnostic marker for non-small cell lung cancer-related malignant pleural effusions. J Cancer Res Clin Onco, Dec 16, 2008 [Epub ahead of print].
20 Clément G GI, He B, Yagui-Beltrán A, Lin YC, You L, Xu Z, Shi Y, Okamoto J, Benhattar J and Jablons D: Epigenetic alteration of the wnt inhibitory factor-1 promoter occurs early in the carcinogenesis of barrett's esophagus. Cancer Sci *99*: 46-53, 2008.
21 Meng X, Sang S, Zhu N, Lu H, Sheng S, Lee MJ, Ho CT and Yang CS: Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. Chem Res Toxicol *15*: 1042-1050, 2002.
22 Lu H, Meng X, Li C, Sang S, Patten C, Sheng S, Hong J, Bai N, Winnik B, Ho CT and Yang CS: Glucuronides of tea catechins: Enzymology of biosynthesis and biological activities. Drug Metab Dispos *31*: 452-461, 2003.
23 Stresemann C, Brueckner B, Musch T, Stopper H and Lyko F: Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res *66*: 2794-2800, 2006.
24 Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W and Yang CS: Tea polyphenol (–)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res *63*: 7563-7570, 2003.
25 Cheung HW, Ching YP, Nicholls JM, Ling MT, Wong YC, Hui N, Cheung A, Tsao SW, Wang Q, Yeun PW, Lo KW, Jin DY and Wang X: Epigenetic inactivation of chfr in nasopharyngeal carcinoma through promoter methylation. Mol Carcinog *43*: 237-245, 2005.
26 Uematsu K, Kanazawa S, You L, He B, Xu Z, Li K, Peterlin BM, McCormick F and Jablons DM: Wnt pathway activation in mesothelioma: Evidence of dishevelled overexpression and transcriptional activity of beta-catenin. Cancer Res *63*: 4547-4551, 2003.
27 Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B and Clevers H: Constitutive transcriptional activation by a beta-catenin-tcf complex in apc–/– colon carcinoma. Science *275*: 1784-1787, 1997.
28 Miller JR: The wnts. Genome Biol. 3:REVIEWS3001, 2002.
29 van Gijn ME, Daemen MJ, Smits JF and Blankesteijn WM: The wnt-frizzled cascade in cardiovascular disease. Cardiovasc Res *55*: 16-24, 2002.
30 Yang CS, Maliakal P and Meng X: Inhibition of carcinogenesis by tea. Annu Rev Pharmacol Toxicol 42: 25-54, 2002.
31 Lambert JD, Hong J, Yang GY, Liao J and Yang CS: Inhibition of carcinogenesis by polyphenols: Evidence from laboratory investigations. Am J Clin Nutr *81*: 284S-291S, 2005.
32 Yang CS, Lambert JD, Hou Z, Ju J, Lu G and Hao X: Molecular targets for the cancer preventive activity of tea polyphenols. Mol Carcinog *45*: 431-435, 2006.
33 Fang M, Chen D and Yang CS: Dietary polyphenols may affect DNA methylation. J Nutr *137*: 223S-228S, 2007.
34 Jian L, Xie LP, Lee AH and Binns CW: Protective effect of green tea against prostate cancer: A case-control study in southeast china. Int J Cancer *108*: 130-135, 2004.

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