# Epigenetic alteration of the Wnt inhibitory factor-1 promoter occurs early in the carcinogenesis of Barrett's esophagus

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**The role of Wnt antagonists in the carcinogenesis of esophageal adenocarcinoma (EAC) remains unclear. We hypothesized that downregulation of the Wnt inhibitory factor-1 (*WIF-1*) might be involved in the neoplastic progression of Barrett's esophagus (BE). We analyzed the DNA methylation status of the *WIF-1* promoter in normal, preneoplastic, and neoplastic samples from BE patients and in EAC cell lines. We investigated the role of WIF-1 on EAC cell growth and the chemosensitization of the cells to cisplatin. We found that silencing of *WIF-1* correlated with promoter hypermethylation. EAC tissue samples showed higher levels of *WIF-1* methylation compared to the matched normal epithelium. In addition, we found that *WIF-1* hypermethylation was more frequent in BE samples from patients with EAC than in BE samples from patients who had not progressed to EAC. Restoration of WIF-1 in cell lines where *WIF-1* was methylation-silenced resulted in growth suppression. Restoration of WIF-1 could sensitize the EAC cells to the chemotherapy drug cisplatin. Our results suggest that silencing of *WIF-1* through promoter hypermethylation is an early and common event in the carcinogenesis of BE. Restoring functional WIF-1 might be used as a new targeted therapy for the treatment of this malignancy. (*Cancer Sci* 2008; 99: 46–53)**

BE is an acquired condition in which the normal squamous epithelium in the distal esophagus has been replaced by a metaplastic columnar epithelium, as a complication of chronic gastroesophageal reflux. The clinical significance of this disease is its associated predisposition to EAC, which arises ultimately after progression through a sequence of increasing degrees of dysplasia.[1] Although the incidence of BE-associated EAC in developed countries has been constantly increasing for the last two decades, the reasons for this remain unknown.[2] Despite advances in treatment, EAC is still a highly lethal disease. New therapies based on better understanding of the molecular alterations occurring during the evolution from BE to EAC are therefore in great need.

Growing evidence has implicated aberrant activation of the Wnt signaling pathway in the pathogenesis of a broad range of cancers.[3–10] In the absence of the secreted Wnt molecules, cytosolic β-catenin is phosphorylated by glycogen synthase kinase β in a multiprotein complex including adenomatous polyposis coli, axin, and casein kinase 1. Following phosphorylation, β-catenin is targeted for degradation through the ubiquitin proteosome pathway. Binding of Wnt proteins to their Frizzled membrane receptors triggers the phosphorylation of the cytoplasmic effector Dishevelled. Dishevelled activity alters the glycogen synthase kinase β phosphorylation of the adenomatous polyposis coli–axin complex and therefore the phosphorylation of β-catenin. This results in an increased cytoplasmic pool of β-catenin, which can subsequently translocate to the nucleus, where it binds to members of the TCF/LEF family of transcription factors to promote the expression of TCF-target genes.[11,12]

Modulation of Wnt signaling on the cell surface occurs through two classes of Wnt antagonists based on different mechanisms of action. The first class, including SFRP and WIF-1, binds directly to the Wnt proteins. The second class inhibits Wnt signaling through binding to the Frizzled co-receptor LRP5/6 and comprises the Dickkopf family.[13]

Epigenetic silencing of the *WIF-1* gene has already been described in various types of human cancer,[14–20] including the esophagus. Interestingly, one published study was exclusively based on squamous carcinomas,[21] and in another histology was not specified.[19] This highlights the novelty of this study. We determined to elucidate whether the *WIF-1* gene is silenced by promoter hypermethylation in EAC cell lines and tissue samples. In addition, we established when in the neoplastic progression of BE the methylation of the *WIF-1* promoter occurs. We also evaluated the role of WIF-1 on the growth and chemosensitivity of EAC cells.

## Materials and Methods

**Cell lines and tissue samples.** The human Barrett's-associated adenocarcinoma cell lines TE-7, BIC-1, and SEG-1 were kindly provided by Dr Michael Korn (University of California, San Francisco). OE19 and OE33 cell lines were obtained from the European Collection of Cell Culture (Salisbury, UK). TE-7, OE19, and OE33 cells were routinely cultured in RPMI-1640 medium and BIC-1 and SEG-1 cells in Dulbecco's modified Eagle's medium (DMEM). Both media were supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL). All cells were cultured at 37°C in a humid incubator with 5% $CO_2$.

Fresh esophageal adenocarcinoma tissue (18 samples) and adjacent normal squamous esophageal epithelium (17 samples) from patients undergoing esophagectomy were collected at the time of surgery (University of California, San Francisco) and immediately snap-frozen in liquid nitrogen. All 47 formalin-fixed and paraffin-embedded tissues analyzed in this study were microdissected. They included 15 biopsies from BE mucosa from patients who had not progressed to dysplasia or adenocarcinoma during follow-up from 4 to 10 years, as well as four normal squamous esophageal epithelia, 16 Barrett's mucosa, and 12 EAC from esophagectomy specimens occurring in the context of BE and selected from the files of the Institute of Pathology,

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Abbreviations: 5-azadC, 5-aza-2′-deoxycytidine; BE, Barrett's esophagus; EAC, esophageal adenocarcinoma; LEF, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related proteins 5 and 6; MSP, methylation-specific polymerase chain reaction; MS-SSCA, methylation-sensitive single-strand conformation analysis; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; SFRP, secreted Frizzled-related protein; TCF, T-cell factor; WIF-1, Wnt inhibitory factor-1.


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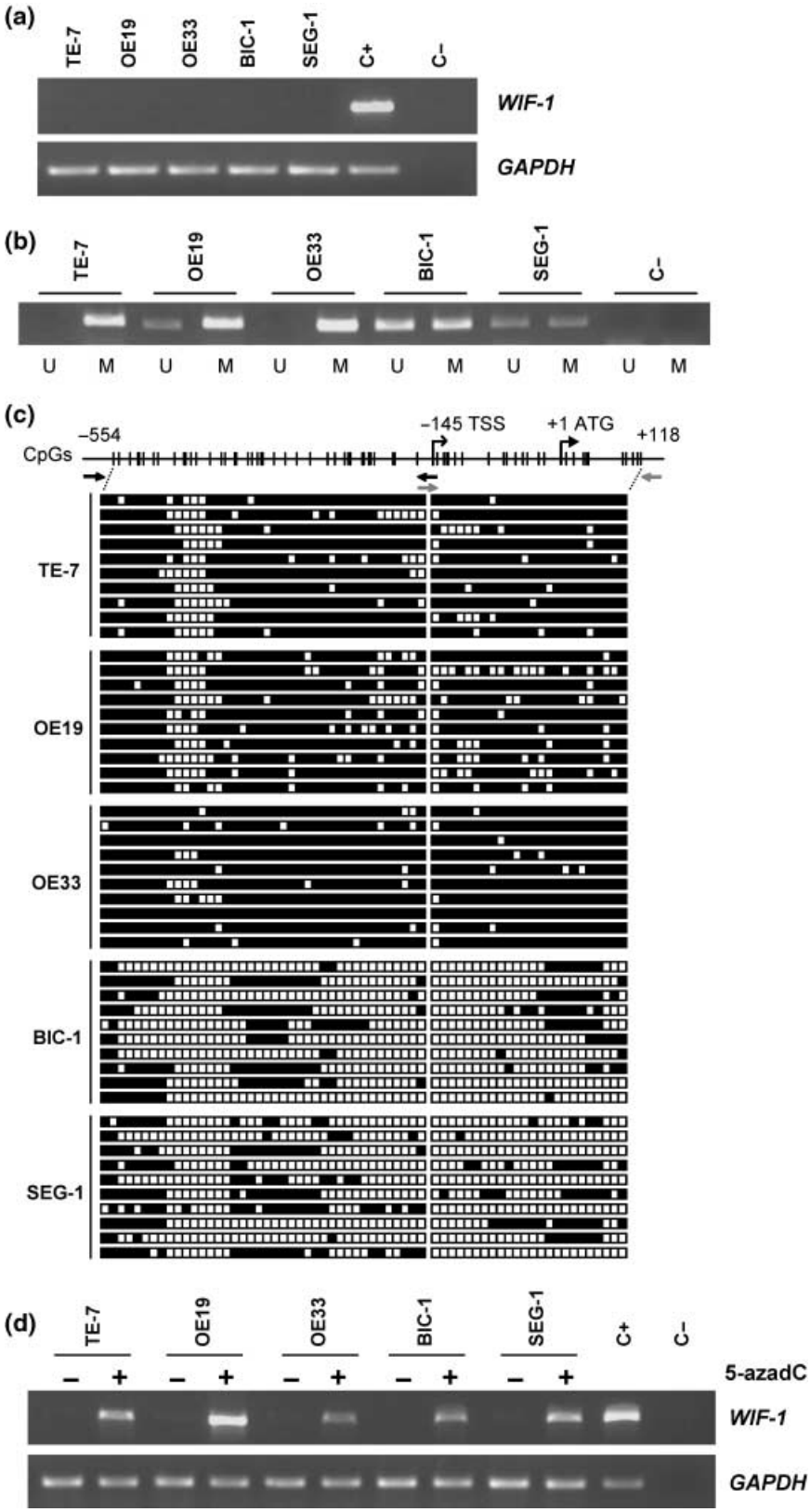


**Fig. 1.** Correlation of methylation in the promoter region with silencing of the Wnt inhibitory factor-1 (*WIF-1*) gene in esophageal adenocarcinoma (EAC) cell lines. (a) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the *WIF-1* gene in five EAC cell lines. The amplified fragment was 451 bp. A 180-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a positive control for RNA quality and loading. C+ and C– corresponded to normal esophagus RNA (BD Biosciences Clontech, Palo Alto, CA) and $H_2O$, respectively, used as a control during the RT-PCR. (b) Methylation-specific polymerase chain reaction analysis of *WIF-1* methylation in EAC cell lines. Bands (198 bp) in lanes labeled "U" are unmethylated DNA product amplified with unmethylation-specific primers. Bands in lanes labeled "M" are methylated DNA product amplified with methylation-specific primers. (c) Bisulfite-sequencing analysis of 64 CpG sites in the region –554 to +118 of the *WIF-1* promoter in EAC cell lines. White squares represent unmethylated CpG sites and black squares indicate methylated CpG sites. Black and gray arrows represent the primers used to amplify the promoter regions –554 to –141 and –161 to +118, respectively. Ten individual clones were analyzed for each cell line. Transcription (TSS) and translation (ATG) start sites are represented (RefSeq NM_007191). (d) Reactivation of *WIF-1* expression by 5-aza-2′-deoxycytidine (5-azadC) treatment in cell lines. RT-PCR analysis of the *WIF-1* gene in EAC cell lines after treatment (2 μM for 6 days) or without treatment with the demethylating agent 5-azadC. The *GAPDH* gene was used as a positive control for RNA quality and loading. C+ and C– corresponded to normal esophagus RNA and $H_2O$, respectively, used as a control during the RT-PCR.

Lausanne, Switzerland. In addition, normal colon mucosa was used as control for the methylation analysis. The use of the human tissues was according to the guidelines of the local ethics committee.

**RNA extraction and RT-PCR.** Total RNA was isolated using an extraction kit (RNeasy Mini Kit; Qiagen, Valencia, CA). RT-PCR was carried out using the SuperScript One-step RT-PCR with Platinum Taq Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Primer sequences for the human *WIF-1* cDNA are: 5′-CCGAAATGGAGGCTTTTGTA-3′ (forward) and 5′-GTGTCTTCCATGCCAACCTT-3′ (reverse). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as an internal control.

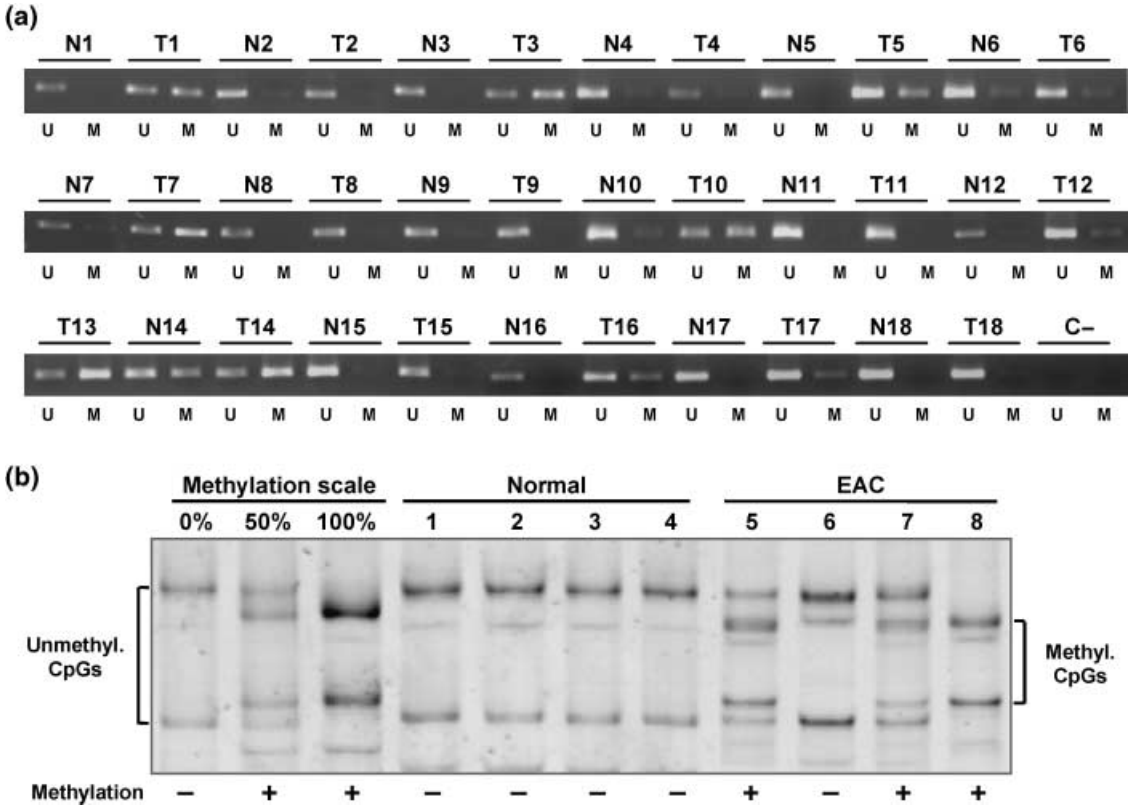


**Fig. 2.** Methylation analysis of the Wnt inhibitory factor-1 (*WIF-1*) promoter in esophageal adenocarcinoma (EAC) tissue samples. (a) Methylation-specific polymerase chain reaction analysis of 17 matched pairs of frozen normal (N) and EAC (T) tissue samples and of one unpaired EAC sample (T13). Bands (198 bp) in lanes labeled "U" are unmethylated DNA product amplified with unmethylation-specific primers. Bands in lanes labeled "M" are methylated DNA product amplified with methylation-specific primers. (b) *WIF-1* promoter methylation by methylation-sensitive single-strand conformation analysis in four microdissected formalin-fixed paraffin-embedded normal squamous epithelia and four EAC samples. Square brackets indicate the migration of either the umethylated DNA (unmethyl. CpGs) or the methylated DNA (methyl. CpGs). + and – indicate the methylation status of each sample.

**DNA extraction and sodium bisulfite conversion.** Genomic DNA of the cell lines and fresh tissue samples was extracted using the Qiagen DNeasy Tissue Kit following the manufacturer's protocol. Bisulfite modification of genomic DNA was carried out using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). Genomic DNA from the formalin-fixed paraffin-embedded tissue sections was extracted after manual microdissection of the lesion of interest as previously described.[22] The total amount of DNA extracted from microdissected tissues was modified in 40 μL of water with sodium bisulfite as previously described.[23]

**MSP, MS-SSCA and sequencing analysis.** Methylation-specific PCR amplifications were carried out using HotStar *Taq* DNA polymerase (Qiagen) for 40 cycles. Primer sequences for the amplification of the methylated DNA are: 5′-GGGCGTTTTATTGGGCGTAT-3′ (forward) and 5′-AAACCAACAATCAACGAAAC-3′ (reverse). Sequences of the unmethylation-specific primers are: 5′-GGGTG-TTTTATTGGGTGTAT-3′ (forward) and 5′-AAACCAACAA-TCAACAAAAC-3′ (reverse). The fragment amplified corresponds to the *WIF-1* promoter region –488 to –291 (the ATG start codon of *WIF-1* was defined as +1).[18] Methylation analysis of DNA extracted from the formalin-fixed paraffin-embedded tissue sections was done by MS-SSCA. The *WIF-1* promoter was amplified by seminested PCR using the following primers: 5′-GCCCGGTAGGTTTTTTGGTATTTAGGT-3′ (forward) and 5′-GCGGCAACCATACTACTCAAAACCTC-3′ (reverse) for the outer PCR and 5′-GGGAATAGTTTTGGTTGAGGGAGTTGT-3′ (forward) for the inner PCR. Forty and 20 cycles were carried out for the outer and inner PCR, respectively. The amplified product includes eight CpG sites between –110 and +6 of the *WIF-1* promoter sequence. Single-strand conformation analysis was carried out as described previously.[23] Bisulfite-treated genomic DNA extracted from EAC cell lines was amplified using two different pairs of primers to amplify nucleotides –554 to +118 of the *WIF-1* promoter region, which covers 64 CpG sites. The sequences of the two pairs of primers used are: 5′-GAGTGATG-TTTTAGGGGTTT-3′ (forward) and 5′-CCTAAATACCAAAAAA-CCTAC-3′ (reverse); and 5′-GTAGGTTTTTTGGTATTTAGG-3′ (forward) and 5′-TCCATAAATACAAACTCTCCTC-3′ (reverse). The PCR products were cloned into the pCR2.1-TOPO Vector using the TOPO TA Cloning Kit (Invitrogen) according to manufacturer's protocol. Ten colonies were randomly chosen for each PCR reaction. Sequencing of the clones was carried out at Quintara Biosciences (Berkeley, CA).

**5-azadC treatment.** EAC cells were seeded in 6-well plates and treated after 24 h with 2 μM 5-azadC (Sigma, St. Louis, MO) in cultured medium for 6 days. Medium was changed after 3 days and fresh 5-azadC was added. Then cells were harvested and genomic DNA and total RNA were extracted. The level of demethylation was checked by MSP and the expression of *WIF-1* was analyzed by RT-PCR.

**TOPflash assay, cell proliferation and cisplatin treatment.** OE19 and BIC-1 cells were plated in a 12-well plate 24 h before transfection. Lipofectamine 2000 (Invitrogen) was used to mediate co-transfection of TOPflash (0.5 μg) or FOPflash (0.5 μg) (vectors were kindly provided by H. Clevers) with a *WIF-1* cDNA construct in pcDNA3.1 vector (1.5 μg)[24] or an empty pcDNA3.1 vector


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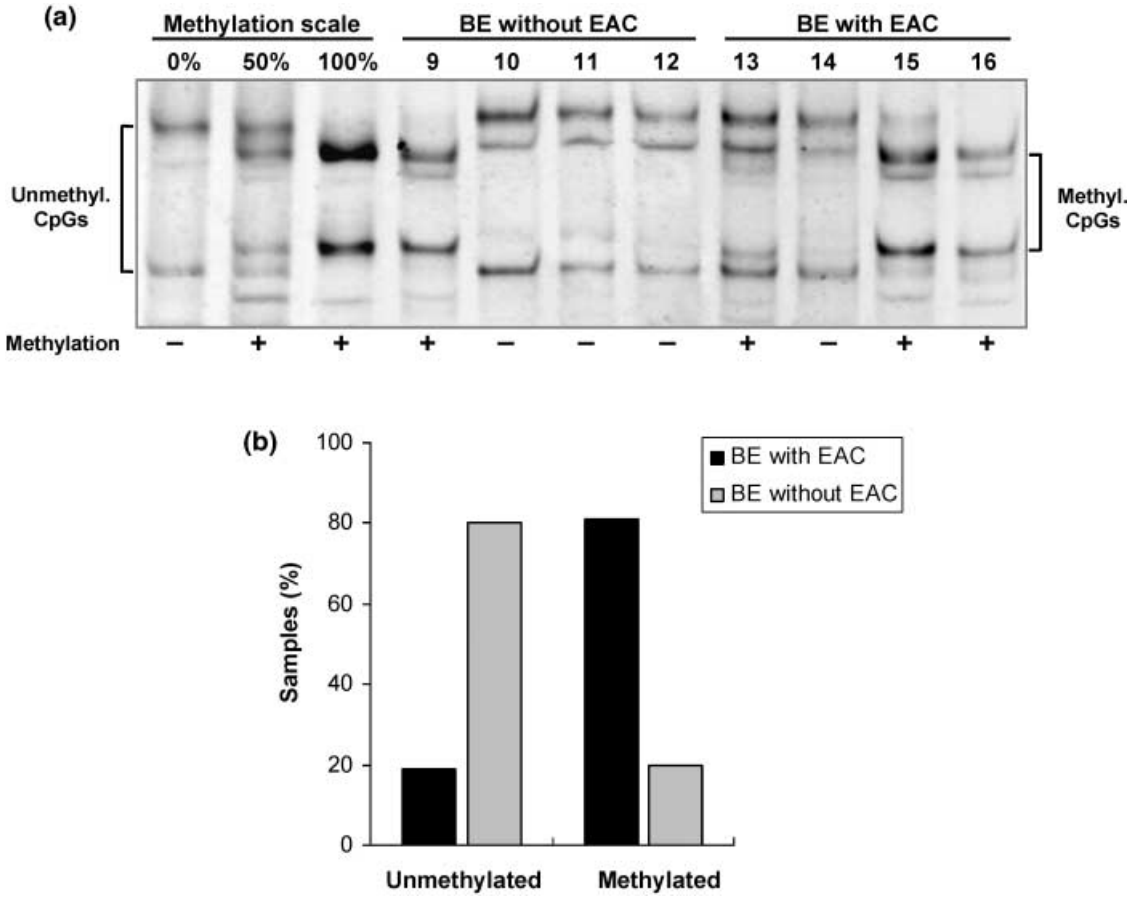


**Fig. 3.** Methylation analysis of the Wnt inhibitory factor-1 (*WIF-1*) promoter in Barrett's esophagus (BE) tissue samples. (a) *WIF-1* promoter methylation by methylation-sensitive single-strand conformation analysis in four BE patients without esophageal adenocarcinoma (EAC) and in four BE patients with EAC. Square brackets indicate the migration of either the umethylated DNA (unmethyl. CpGs) or the methylated DNA (methyl. CpGs). + and – indicate the methylation status of each sample. (b) Graphical representation of the methylated and unmethylated samples of BE without and BE with EAC.

(1.5 μg; Invitrogen). The Renilla luciferase reporter vector (0.05 μg) (Promega, Madison, WI) was simultaneously transfected as the control for transfection efficiency. The expression level of introduced *WIF-1* was analyzed by RT-PCR as described above. As a control for the assay, an *SFRP4* cDNA construct in pcDNA3 vector[25] was co-transfected with the TOPflash or FOPflash in OE19 cells. Cells were harvested 48 h after transfection. Luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega). The experiments were done in triplicate and repeated independently at least four times.

Cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Briefly, OE19 cells were plated in a 6-well plate 24 h before transfection. Transient transfection was carried out using 4 μg of the *WIF-1* cDNA construct or the empty pcDNA3.1 vector. Twenty-four hours after transfection, cells were seeded in a 96-well plate and cultured for a further 24 h before addition of the CellTiter 96 AQueous One Solution (day 0). Cisplatin (Sicor; SICOR Pharmaceuticals, Irvine, CA) was diluted in medium culture at a concentration of 0.1 μg/mL (0.33 μM) at day 0 and added fresh every day. Absorbance was reported at 490 nm. Each experiment was done in sixplicate and repeated at least three times.

**Colony formation assay.** Transfection was carried out with OE19 cells in 6-well plates with 4 μg of *WIF-1* cDNA construct or empty pcDNA3.1 vector as described above. Transfected cells were collected and plated on 10-cm cell culture dishes 24 h after transfection. The cells were then selected by G418 (400 μg/mL). After 10 days, cells were counted and 2000 stable transfected cells were plated on 10-cm cell culture dishes in triplicate. The selection by G418 was maintained for four additional weeks. Colonies were stained by using 0.5% crystal violet and counted.

**Statistical analysis.** Data shown represent mean values (± standard deviation). The statistical significance of differences in levels of methylation between the two groups of BE (BE without EAC *vs* BE with EAC) was established using Fisher's exact test. Student's *t*-test was used for comparing activities of different constructs and treatments.

## Results

**Silencing of *WIF-1* correlates with promoter hypermethylation in EAC cell lines.** We examined *WIF-1* expression in five EAC cell lines (Fig. 1a). *WIF-1* expression was detectable in the normal esophagus control sample, but the transcript was missing in all EAC cell lines. MSP analysis revealed methylation of the *WIF-1* promoter in all the cell lines (Fig. 1b). However, for OE19, BIC-1, and SEG-1 cells both methylated and unmethylated bands were detected. Bisulfite sequencing showed that the CpG islands of TE-7, OE19, and OE33 cell lines were densely methylated (Fig. 1c). Consistent with MSP results, we observed partial methylation of the CpG sites in BIC-1 and SEG-1 cells. We treated the EAC cells with a demethylating agent 5-azadC and found that *WIF-1* expression was restored after the treatment (Fig. 1d). Taken together, these results suggest that the expression of *WIF-1* in EAC is regulated by hypermethylation of its promoter.

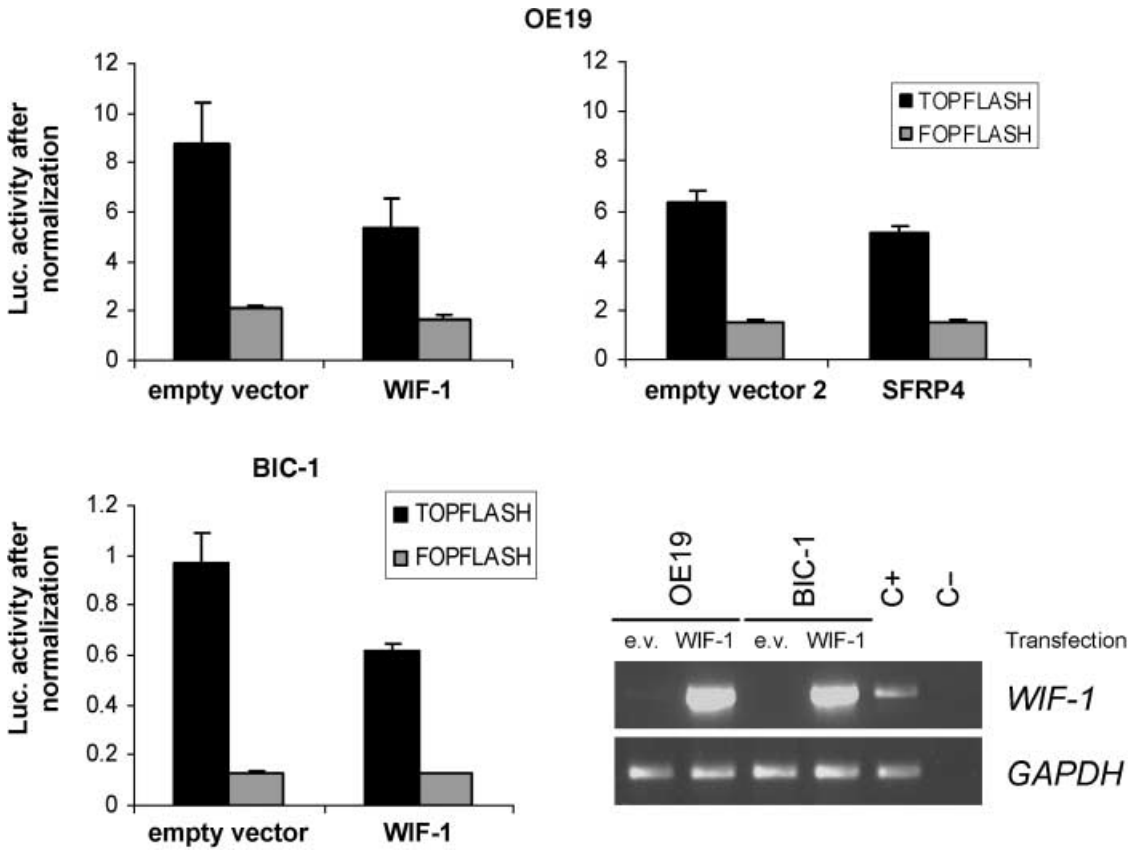


**Fig. 4.** Effect of the restoration of Wnt inhibitory factor-1 (*WIF-1*) expression on the Wnt-dependent transcription activity. OE19 and BIC-1 cells were co-transfected with 1.5 μg of *WIF-1* expression vector or empty vector (pcDNA3.1) and 0.5 μg of TOPflash or FOPflash reporter. As a positive control, OE19 cells were cotransfected with 1.5 μg of *SFPR4* expression vector or empty vector 2 (pcDNA3) and 0.5 μg of TOPflash or FOPflash reporter. Firefly luciferase activity of the reporters is represented after normalization of each sample with the Renilla luciferase activity. The experiment was carried out in triplicate. The expression level of *WIF-1* in OE19 and BIC-1 cells was detected by reverse transcription–polymerase chain reaction (RT-PCR) after transfection of the empty vector (e.v.) or *WIF-1* expression vector. The amplified fragment was 451 bp. A 180-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a positive control for RNA quality and loading. C+, normal esophagus RNA; C–, $H_2O$, used as a control during RT-PCR.

***WIF-1* promoter is methylated in EAC tissue samples.** We investigated the methylation status of the *WIF-1* promoter in 18 frozen EAC tissue samples (17 were matched pairs of normal and tumor tissue). We found aberrant methylation of the *WIF-1* promoter in 11/18 tumor samples (61%), whereas methylation was not detected in seven matched pairs of normal and tumor samples (Fig. 2a). Cases 6, 7, 10 and 14 showed some methylation in both tumor and their matched normal samples. This might be due to contamination of tumor cells in the normal specimen or to premalignant changes of the squamous epithelium adjacent to EAC. Similarly, MSP revealed unmethylated bands in every methylated tumor sample. This could be interpreted as contamination of normal cells in the tumor specimens or partial methylation of the *WIF-1* promoter (mixture of methylated and unmethylated alleles).

In addition, we examined the methylation status of the *WIF-1* promoter in 12 microdissected formalin-fixed paraffin-embedded EAC specimens and four adjacent normal squamous epithelia using MS-SSCA (Fig. 2b). The methylation scale was carried out as described previously.[26] None of the normal squamous epithelia were methylated. Ten of 12 (83%) microdissected tumor samples were methylated for the *WIF-1* promoter. Among those, three showed full methylation, and seven had unmethylated and methylated *WIF-1* alleles at varying ratios. A mixture of methylated and unmethylated alleles can be explained by either monoallelic methylation of the *WIF-1* promoter or by the existence of subpopulations of cells, some with methylation of the *WIF-1* promoter and some without.

**Hypermethylation of the *WIF-1* promoter occurs early in the carcinogenesis of EAC.** We further analyzed the methylation status of the *WIF-1* promoter in the premalignant lesions of EAC. Fifteen microdissected formalin-fixed paraffin-embedded BE samples from patients who had not progressed to dysplasia or EAC, as well as 16 non-dysplastic microdissected fixed BE samples adjacent to EAC were subjected to MS-SSCA (Fig. 3a,b). Methylation was detected in 3/15 (20%) BE samples from patients without EAC. Among those three samples, one showed complete CpG island methylation of the *WIF-1* promoter and the other two a mixture of methylated and unmethylated alleles. In contrast, *WIF-1* promoter hypermethylation was found in 13/16 (81%) BE samples from patients with EAC. Among those samples, four showed complete methylation of the *WIF-1* promoter, and nine had a mixture of methylated and unmethylated *WIF-1* alleles. Statistical analysis of the difference between the methylated and unmethylated samples in the two groups of BE (BE without EAC *versus* BE with EAC) was highly significant ($P < 0.001$).

***WIF-1* expression modulates the Wnt signaling pathway in EAC cell lines.** We subsequently restored *WIF-1* expression in the OE19 and


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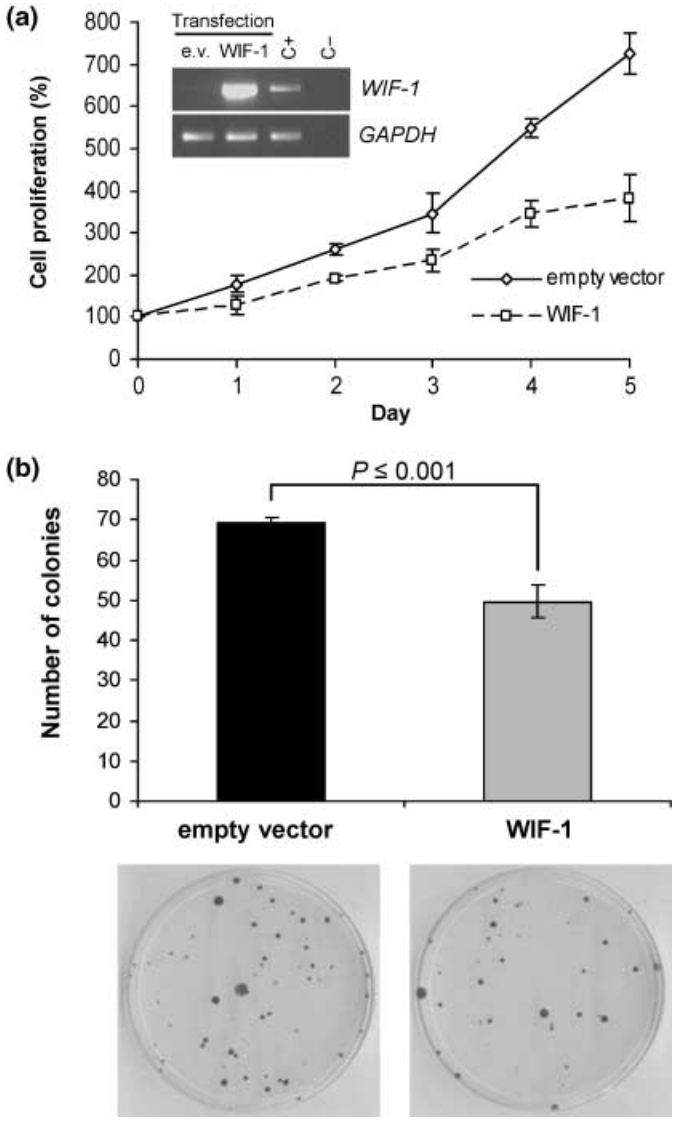


**Fig. 5.** Growth suppression of OE19 cells by restoration of Wnt inhibitory factor-1 (*WIF-1*) expression. (a) Cell proliferation of OE19 cells after transfection of *WIF-1* expression vector or empty vector. Measurements were carried out 2 days after transfection (day 0) and repeated during the following five consecutive days. Results are means of sixplicates with error bars (standard deviation [SD]). Expression level of introduced *WIF-1* was detected by reverse transcription–polymerase chain reaction as for Fig. 4. (b) Colony formation assay using OE19 cells. The cells were transfected with empty vector or *WIF-1* expression vector, selected with G418 for 10 days and 2000 cells were plated in triplicate in 10-cm dishes. Selection was maintained for four additional weeks. The bar graph represents the average of colony numbers of the triplicated experiments. Error bars are SD.

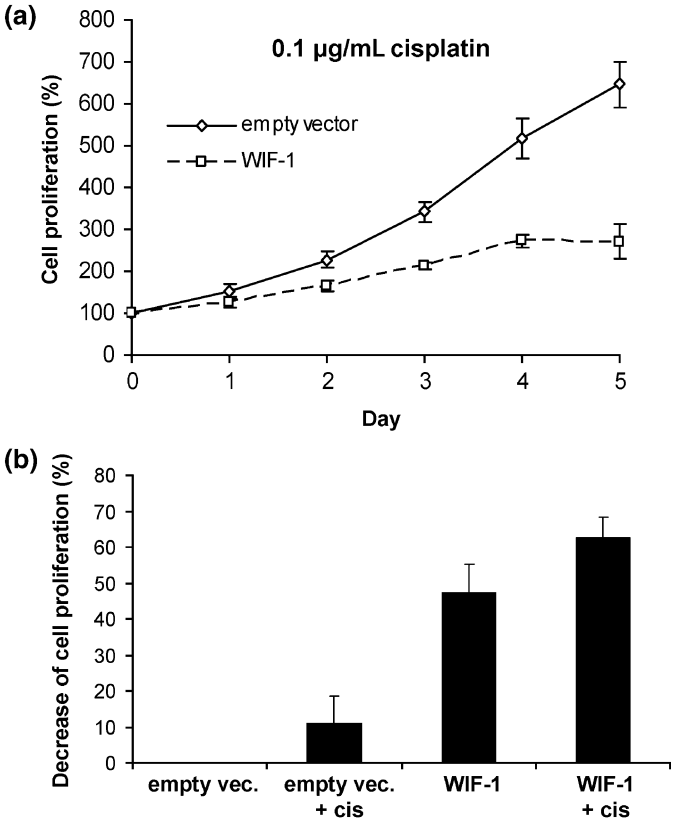


**Fig. 6.** Growth suppression of OE19 cells by restoration of Wnt inhibitory factor-1 (*WIF-1*) expression and treatment with the chemotherapy drug cisplatin. (a) Cell proliferation of OE19 cells after transfection of *WIF-1* expression vector or empty vector. Cells were subjected to cisplatin 2 days after transfection (day 0) and measurements of the cell proliferation were repeated during the following five consecutive days. Cisplatin was added fresh every day. Results are means of sixplicates with error bars (standard deviation). (b) Graphical representation of the cell proliferation decrease at day 5 for each treatment.

BIC-1 cell lines by transiently transfecting a *WIF-1* expression vector and analyzed the Wnt signaling pathway activity by simultaneously co-transfecting the TOPflash vector. RT-PCR analysis confirmed that *WIF-1* was overexpressed in the cells transiently transfected with the *WIF-1* expression vector compared to the cells transfected with the empty vector pcDNA3.1 (Fig. 4). Restoration of WIF-1 was able to decrease the TOPflash activity by approximately 39% and 36% in OE19 and BIC-1 cells ($P < 0.05$), respectively, compared to the TOPflash activity after co-transfection with the empty expression vector pcDNA3.1 (Fig. 4). To validate the accuracy of this assay in our cell lines, the TOPflash vector was co-transfected with an *SFRP4* expression vector in OE19 cells that lacked *SFRP4* expression (data not shown), because restoration of SFRP4 has been shown to modulate the Wnt signaling pathway.[27] As expected, restoration of SFRP4 decreased the TOPflash activity by approximately 20%. As a negative control for this assay, we confirmed that forced expression of *SOCS-3*, a Wnt signaling independent factor,[28] did not modulate the Wnt signaling pathway (data not shown).

**Restoration of *WIF-1* suppresses EAC cell growth.** We carried out a cell proliferation assay after transient transfection with a *WIF-1* expression vector in OE19 cells. One week after transfection, the proliferation of the empty vector transfected OE19 cells reached 725% (100% corresponded to the cell proliferation at day 0), whereas the proliferation of the *WIF-1* transfected cells was 381% (approximately 47% decrease) (Fig. 5a).

In addition, after selection of G418-resistant colonies, we found that the colony numbers of *WIF-1* transfected OE19 cells significantly decreased compared to that of empty vector transfected cells ($P \leq 0.001$) (Fig. 5b). Taken together, our data indicate that restoration of WIF-1 suppresses EAC cell growth.

**Restoration of *WIF-1* sensitizes EAC cells to the chemotherapy drug cisplatin.** Lastly, we exposed the cells to cisplatin, a drug commonly used for the treatment of EAC patients, after transient transfection of a *WIF-1* expression vector. After 1 week of transfection and exposure to 0.1 µg/mL cisplatin, the cell proliferation of the empty vector transfected cells was higher than that of *WIF-1* transfected cells (646% *vs* 271%) (Fig. 6a).

Most interestingly, *WIF-1* restoration conjugated to cisplatin treatment decreased cell growth (approximately 62%) more than WIF-1 restoration or cisplatin treatment alone (47% and 11%, respectively) ($P < 0.05$), suggesting that WIF-1 might sensitize EAC cells to cisplatin treatment (Fig. 6b).

## Discussion

Numerous studies have established the crucial role of the Wnt signaling pathway in the pathogenesis of many human cancers, including the finding of nuclear accumulation of β-catenin early in the transition from BE to EAC.[3,29] Likewise, overexpression of Wnt signaling components as well as downregulation of Wnt antagonist *SFRP* have been confirmed along the EAC carcinogenesis sequence.[10,30]

In the present study, we investigated the methylation status of one of the Wnt antagonists *WIF-1* and its role in EAC. We found that *WIF-1* is downregulated in EAC cell lines and that frequent dense hypermethylation of the *WIF-1* promoter region is correlated with its silencing. Similar to the cell lines, *WIF-1* promoter hypermethylation was also commonly found in EAC tissue samples. Taken together, our data suggest that aberrant hypermethylation of the *WIF-1* promoter is a frequent epigenetic event leading to *WIF-1* silencing in EAC.

The majority of EAC is diagnosed at an advanced stage, and is associated with poor prognosis. Only early detection of cancer or preneoplastic lesions will allow effective treatment resulting in decreased mortality. Currently it is not possible to predict which BE patients will progress through dysplasia to EAC. Because the *WIF-1* promoter is frequently methylated and occurs early during the neoplastic progression of BE (we found *WIF-1* promoter methylation in 81% of BE patients with EAC but in only 20% of the BE patients without EAC), *WIF-1* promoter hypermethylation could be used as a marker to distinguish progressive from non-progressive BE. However, confirmation in a larger cohort of samples is needed before it can be used clinically. Interestingly, the *SFRP1* promoter was hypermethylated in BE patients with EAC as well as in BE patients without EAC.[31] Therefore, we hypothesize that SFRP1 and WIF-1 might have distinct roles in EAC carcinogenesis. Epigenetic alterations of the *SFRP1* promoter might be associated with dysregulation of differentiation, leading to BE; epigenetic alterations of the *WIF-1* promoter might be associated with EAC carcinogenesis.

We showed that restoration of WIF-1 in EAC cell lines lacking *WIF-1* expression decreased the activity of the Wnt/β-catenin signaling pathway. The situation in EAC tissue samples seems more controversial. To date, several studies have reported Wnt signaling activation, indicated by nuclear β-catenin accumulation, only in the presence of low-grade dysplasia.[29,32] Nevertheless, we observed that promoter hypermethylation of the *WIF-1* gene had already occurred in BE samples. Although those studies were carried out by several groups using different tissue samples, the data might indicate either that downregulation of *WIF-1* alone is not sufficient for the activation of the pathway, or that other concurrent alterations (overexpression of specific Wnt ligands and/or mutations of downstream components) are necessary to activate the pathway during BE neoplastic progression. It is also possible that WIF-1 is involved in other pathways. Recently, Ohigashi *et al*. showed that WIF-1 downregulates the PI3K/Akt pathway,[33] supporting this hypothesis. In addition, the WIF domain shares homology with the extracellular domain of the RYK family of receptor tyrosine kinases.[13,34]

Targeted inhibition of Wnt signaling has been shown to induce apoptosis and inhibit cancer cell growth.[7,24,25,27,35–37] When we restored *WIF-1* expression in EAC cell lines in which *WIF-1* was methylation silenced, cell proliferation and colony formation were inhibited. Furthermore, we showed that restoration of WIF-1 in cells lacking *WIF-1* expression sensitize the cancer cells to the chemotherapy drug cisplatin. Taken together, our findings suggest that restoration of WIF-1 alone or in combination with traditional chemotherapy drugs might offer a new targeted therapy for the treatment of EAC patients.

In conclusion, this study highlights a new epigenetic event in the carcinogenesis of EAC. Aberrant *WIF-1* promoter hypermethylation appears to occur early in the neoplastic progression of BE. Methylation of *WIF-1* promoter was observed more frequently in BE patients with EAC than in BE patients who had not progressed to cancer. Therefore, epigenetic alteration of *WIF-1* could be used as a new diagnostic and predictive marker for increased EAC risk in BE patients. Finally, the high prevalence of *WIF-1* hypermethylation in EAC patients supports the potential development of novel therapies exploiting the restoration of *WIF-1* function.

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