Wnt Inhibitory Factor-1 Is Silenced by Promoter Hypermethylation in Human Lung Cancer

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Abstract

Aberrant activation of the Wingless-type (Wnt) signaling pathway is associated with a variety of human cancers, and we recently reported the importance of aberrant Wnt signaling in lung cancer. On the other hand, inhibition of Wnt signaling suppresses growth in numerous cell types. Wnt inhibitory factor-1 (WIF-1) is a secreted antagonist that can bind Wnt in the extracellular space and inhibit Wnt signaling. Recently, down-regulation of WIF-1 has been reported in several human cancers. To discover the mechanism of WIF-1 silencing in lung cancer, we first identified the human WIF-1 promoter and subsequently examined the methylation status in the CpG islands. By using methylation-specific PCR and sequence analysis after bisulfite treatment, we demonstrate here frequent CpG island hypermethylation in the functional WIF-1 promoter region. This hypermethylation correlates with its transcriptional silencing in human lung cancer cell lines. Moreover, treatment with 5-aza-2′-deoxycytidine restores WIF-1 expression. We then studied WIF-1 expression in 18 freshly resected lung cancers, and we show a down-regulation in 15 of them (83%). This silencing also correlates with WIF-1 promoter methylation. Our results suggest that methylation silencing of WIF-1 is a common and likely important mechanism of aberrant activation of the Wnt signaling pathway in lung cancer pathogenesis, raising its therapeutic interest.

Introduction

The Wingless-type (Wnt) family of secreted glycoproteins is a group of signaling molecules that is widely involved in developmental processes and oncogenesis (1, 2). The proto-oncogenic effects of Wnt were discovered more than 20 years ago (3), and since then, numerous reports have demonstrated aberrant activation of the Wnt signaling pathway in disparate human cancers such as colorectal cancer (4), head and neck carcinoma (5), melanoma (6), and leukemia (7). Recently, we reported the overexpression of disheveled (Dvl) proteins in mesothelioma and non-small cell lung cancer (NSCLC) (8, 9), and we demonstrated that inhibition of Wnt-1 induces apoptosis and inhibits tumor growth in lung cancer cell lines (10).

Wnt antagonists can be divided into two groups according to the mechanisms of their functions: The first group includes the secreted frizzled-related protein (sFRP) family, Wnt inhibitory factor-1 (WIF-1), and Cerberus. They inhibit Wnt signaling by direct binding to Wnt molecules. The second group, including the Dickkopf (Dkk) family, inhibits Wnt signaling by binding to the LRPS/LRP6 component of the Wnt receptor complex (11). These inhibitors have been studied extensively in developmental studies. Recently, their involvement in oncogenesis has been demonstrated. For example, loss of expression of the sFRP family has been reported in cervical carcinomas (12), breast cancers (13), and gastric cancers (14); and the sFRP promoter has been shown methylated in colorectal tumorigenesis (15–17).

WIF-1 is a highly conserved gene first identified from the human retina. WIF-1 does not share any similarities with the cysteine-rich domain of Fz or sFRP (18). WIF-1 has an NH2-terminal signal sequence, a unique WIF domain, and five epidermal growth factor-like repeats. Overexpression of WIF-1 in X. laevis embryos blocks the Wnt-8 pathway and induces abnormal somitogenesis (19). Recently, Wissmann et al. (20) reported the down-regulation of WIF-1 in several cancer types including lung cancer using a chip hybridization assay and immunohistochemistry.

To understand further the role that WIF-1 plays in Wnt signaling in human cancers, we cloned the WIF-1 promoter. We then investigated the expression and the epigenetic regulation of WIF-1. We found that WIF-1 was frequently silenced by hypermethylation of its promoter in lung cancer cell lines as well as in fresh lung cancer tissues, suggesting that WIF-1 is an important player in the involvement of the Wnt pathway in carcinogenesis and particularly in lung cancer.

Materials and Methods

Cell Lines. NSCLC cell lines (NCI-H1703, NCI-H460, NCI-H838, and NCI-A549) were obtained from American Type Culture Collections (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Normal human small airway epithelial cells and bronchial epithelial cells (NHBE, 16BHE; primary cultures) were obtained from Clonetics (Walkersville, MD) and cultured in Clonetics SAGM Bullet kit. All cells were cultured at 37°C in a humid incubator with 5% CO2.

Tissues Samples. Fresh lung cancer tissues and adjacent normal lung tissues from patients undergoing resection for lung cancers were collected at the time of surgery and immediately snap-frozen in liquid nitrogen (Institutional Review Board approval H8714-15319-040). These tissue samples were kept at −170°C in a liquid nitrogen freezer before use.

Reverse Transcription-PCR. Total RNA from lung cancer cell lines, fresh lung cancer, and paired adjacent normal tissue was isolated using an extraction kit (RNAasy Mini kit; Qiagen, Valencia, CA). Reverse transcription-PCR was performed in GeneAmp PCR system 9700 using One-step reverse transcription-PCR kit from Life Technologies, Inc., according to the manufacturer’s protocol. Primers for reverse transcription-PCR were obtained from Operon Technologies, Inc. (Alameda, CA). Primer sequences for the human WIF-1 cDNA were 5′-CCGAAAATGGACGCTTTTGA-3′ (forward) and 5′-TGTGGACGATTTGTCGTTTTG-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. 5-Aza-2′-deoxycytidine (Sigma, St. Louis, MO) treatment was performed as described previously (21).

Sequencing Analysis. Genomic DNA of the cell lines and fresh tissue samples was extracted using DNA STAT-60 reagent (TEL-TEST, Inc., Friendswood, TX), according to the manufacturer’s protocol. Bisulfite modification of
Identification of the WIF-1 Promoter Region. To identify the WIF-1 promoter, we conducted a BLAST search with the 1140-bp coding sequence of WIF-1 as a virtual probe against the human genomic database at the University of California, Santa Cruz, web server. We used a promoter search program to confirm that the 5’ region of the gene presents classical features of a promoter region. We then used a CpG island search program to map the CpG islands within the WIF-1 promoter (Fig. 1). We found 105 CpGs in this promoter region (1.2 kb before the ATG of the WIF-1 open reading frame).

WIF-1 is Silenced by Promoter Hypermethylation in Human Lung Cancer Cell Lines. We first examined WIF-1 expression in several normal and tumor cell lines by using semiquantitative reverse transcription-PCR (Fig. 2A). We found that WIF-1 was expressed in all three normal primary cell cultures, NHBE, 16 HBE, and small airway epithelial cells. In contrast, the WIF-1 transcript was missing or dramatically underexpressed in three of four NSCLC cell lines. We next analyzed the methylation status of the CpG islands within these cell lines. All cancer cell lines tested lacking WIF-1 expression were found to be hypermethylated using MSP (Fig. 2B). In contrast, no hypermethylation was seen in all normal controls that expressed WIF-1. WIF-1 was underepressed to a lesser extent in H1703 cell line, and MSP only shows partial methylation in this cell line. We also used bisulfite sequencing to analyze details of the methylation status of 60 CpG sites in the 672-bp fragment of WIF-1 including the promoter from −554 to ATG and a part of the first exon from ATG to +118 in several cell lines (Fig. 3A). Consistent with MSP results, we found that these CpG islands were densely methylated in all of the NSCLC cell lines tested. In addition, we found that the WIF-1 expression was restored after the demethylating agent 5-aza-2’-deoxycytidine treatment in those cell lines lacking WIF-1 expression (Fig. 3B).

These results suggest that the status of WIF-1 expression in NSCLC cell lines correlates with dense CpG methylation of the WIF-1 promoter region.

WIF-1 is Silenced by Promoter Hypermethylation in Fresh Human NSCLC Tissue Samples. Next, we analyzed the WIF-1 expression and methylation status in primary NSCLC tissue samples. Among 18 matched pairs of surgically resected early stage lung cancers we examined, 15 cancer samples (83%) were found to have no or little WIF-1 mRNA compared with their autologous normal samples (Fig. 4A). By using MSP, we found aberrant methylation in all tumor samples that lack WIF-1 expression but not in their matched normal samples (Fig. 4B). This correlation was demonstrated in eight cases for which both RNA and bisulfite-treated genomic DNA were available (patients 6, 7, 9, 10, 11, 12, 15, and 18). We also performed MSP in eight additional matched samples (patients 19–26) and found evidence of methylation in seven of them. In several cases, slight methylation as well as unmethylated bands were observed, respectively, in normal and tumor tissues, probably due to unavoidable contamination of cancer cells in the noncancer specimen or premalignant changes of peritumoral normal tissue. These data indicate that silencing of WIF-1 is related with hypermethylation of its promoter in primary NSCLC tissue samples. In addition, we analyzed sequences of the WIF-1 promoter region after bisulfite treatment for eight samples. We detected dense methylation in those CpG sites (two

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In summary, WIF-1 is frequently down-regulated in NSCLC, and this down-regulation is correlated with the promoter hypermethylation.

Discussion

Members of the Wnt pathway have been shown to play a critical role in human carcinogenesis. Overexpression of Wnt genes has been reported in many cancers, and we described a role for Wnt signaling in NSCLC and mesothelioma (8–10). Wnt antagonists have been recently identified, and their role in carcinogenesis is becoming more clearly understood. WIF-1 is known to bind Wnt proteins and to inhibit their activities (19). Recently, Wissman et al. (20) reported by using a microarray analysis the down-regulation of WIF-1 in several human cancers and confirmed the latter by immunohistochemistry in 60% of breast cancers and 75% of lung cancers. Here, we report the down-regulation of WIF-1 in NSCLC cell lines at a transcriptional level. Moreover, we find that WIF-1 was also down-regulated in 83% of fresh NSCLC surgical specimens when compared with normal
tissue. WIF-1 silencing correlates with its promoter hypermethylation in both cancer cell lines and human NSCLC primary tissues. We propose that WIF-1 down-regulation occurs in NSCLC through an epigenetic regulation as previously reported for sFRP in colorectal carcinoma (16). It is noteworthy that WIF-1 lacks any sequence similarity with the cysteine-rich domain of Fz or sFRP (18). WIF-1 contains a WIF domain and five epidermal growth factor-like repeats. It can bind to XWnt-8 and Drosophila Wg in the extracellular space and inhibit XWnt-8-Dfz interactions (19). The mechanism by which WIF interacts with Wnt remains partially understood. We identified Tcf-responsive elements in the WIF-1 promoter (data not shown), suggesting that WIF-1 can act as a negative feedback regulator of Wnt signaling.

Aberrant methylation of promoter regions that silences transcription of the genes has been recognized as a mechanism for inactivating tumor suppressor genes in human cancer (22, 23). In lung cancer, p16 was first reported to be methylated (24). Now, many other genes such as APC, H-Cadherin, Glutathione S-Transferase, Retinoic acid receptor β-2, E-Cadherin, and RAS association domain family IA (25) have been shown to be methylated in various types of lung cancer, and we recently reported the hypermethylation of the human SOCS-3 promoter in NSCLC (21). Our findings of WIF-1 silencing by promoter methylation reveal an important epigenetic event during the development of NSCLC, suggesting that WIF-1 may be a key antagonist of Wnt signaling in lung cancer.

Taken together, our results suggest that WIF-1 silencing occurs as a result of promoter hypermethylation and may be an important cause of constitutive activation of the Wnt pathway in cancer and especially in lung cancer. Our findings reinforce the therapeutic potential of inhibiting the Wnt signaling pathway through such strategies as anti-body blockade and raise the interest of reversing WIF-1 inactivation by demethylating agents. Such strategies would be of particular interest in NSCLC for which the Wnt pathway appears to be of paramount importance and current treatments remain disappointing.

References
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