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Cloning and characterization of the promoter of human Wnt inhibitory factor-1

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Abstract

Wnt inhibitory factor-1 (WIF-1) is a secreted antagonist of Wnt signaling and functions by directly binding to Wnt ligands in the extracellular space. Here we report the identification of the 5′ promoter region (~1.5 kb) of the human WIF-1 gene. Functional analysis of this region shows that a whole fragment displays high basal promoter activity in different cell lines, while the truncated forms do not, indicating that integrity of the WIF-1 promoter region may be important for WIF-1 activity. Moreover, we found that the expression level of β-catenin in cancer cell lines correlates with the WIF-1 promoter activity, suggesting that the WIF-1 promoter may be regulated by the Wnt/β-catenin pathway and may function in a negative feedback manner. Our results also suggest that a methylated CpG island, which we observed recently in human lung cancer, lies within the functional WIF-1 promoter region and therefore bears the importance of the methylation-status of this CpG island as an important key in Wnt activation in human cancer.

Keywords: WIF-1; Promoter; Luciferase reporter; Wnt/β-catenin signaling; Cancer

The Wnt protein family consists of at least 19 members [1,2]. These secreted glycoproteins are signaling molecules widely involved in developmental processes and oncogenesis [3,4]. Aberrant activation of the Wnt signaling pathway has been demonstrated in a variety of human cancers such as colorectal cancer [5], head and neck carcinoma [6], melanoma [7], and leukemia [8]. We recently reported that Wnt activation is associated with overexpression of Disheveled (Dvl) proteins in mesothelioma and non-small-cell lung cancer (NSCLC) [9,10] and that blocking Wnt-1 signaling induces apoptosis and suppresses growth in NSCLC and mesothelioma cells [11,12].

Two groups of Wnt antagonists with different mechanisms of action have been identified so far [1]. The first group, which includes the secreted frizzled-related protein (sFRP) family, Wnt inhibitory factor (WIF)-1, and Cerberus, inhibits Wnt signaling by directly binding to Wnt molecules. The second group consists of the Dickkopf (Dkk) family, which inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex [1]. Involvement of the sFRP family in oncogenesis has recently been reported. Loss of expression of sFRP family proteins has been found in cervical carcinomas [13], breast cancer [14], and gastric cancer [15]. The sFRP promoter has been found methylated in colorectal cancer and restoration of sFRPs in colon cancer cells results in apoptosis and suppression of Wnt-dependent transcription [16–18].

The role of WIF-1 in Wnt signaling was first identified in the human retina with highly conserved orthologues in

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Xenopus and zebrafish [19]. WIF-1 is an evolutionarily conserved protein located at chromosome 12q14 which contains an N-terminal signal sequence, a unique WIF domain, and five-epidermal growth factor (EGF)-like repeats. It does not share any similarities with the cysteine-rich domain (CRD) of frizzled (Fz) or sFRP [20]. It has been shown that WIF-1 can bind to XWnt-8 and *Drosophila* wingless (Wg) in the extracellular space and inhibit Xwnt-8–Dfz2 interactions and Armadillo stabilization in Xwnt-8-treated *Drosophila* clone-8 cells [19]. Wissman et al. [21] reported the downregulation of WIF-1 in several cancer types including lung cancer, however the role that WIF-1 plays in human cancers still remains unclear. We recently investigated the expression and the epigenetic regulation of WIF-1 in lung cancer. We found WIF-1 underexpression in NSCLC cell lines and tissue samples and this silencing correlates with the methylation of the CpG island located in the 5′ flanking region of the WIF-1 gene [22].

To further understand the involvement of WIF-1 in Wnt signaling, we report in this study the cloning and characterization of the human WIF-1 promoter. We found different activities of the WIF-1 promoter in different human cancer cell lines and the level of promoter activity is correlated with the expression level of cytosolic β-catenin. Moreover, we demonstrate that the CpG island is within the functional promoter of the WIF-1 gene. Therefore, methylation in this regulatory region may be an important mechanism of aberrant Wnt signaling activation in cancer.

Materials and methods

Cell cultures. Human kidney transfected epithelial cell line (293T), human non-small-cell lung cancer cell line (NCI-A549), human colorectal cancer cell lines (HCT116 and SW480), which harbor mutations in CTNNB1 and APC, respectively [23,5], and human mesothelioma cancer cell lines (H28, MS1) that lack β-catenin expression [12] were obtained from American Type Culture Collections (ATCC, Manassas, Virginia). These cells, except human kidney transfected epithelial cell line (293T), were cultured in RPMI 1640. Human kidney epithelial cell line (293T) was cultured in Dulbecco's modified Eagle's medium (DME H-21). All cells were supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humid incubator with 5% CO_2.

Cloning and sequence analysis. We used a PCR-based technique to clone the human WIF-1 promoter region. Sense and anti-sense primers were chosen from the genomic sequence of the human WIF-1 gene (Accession No. NM007191), corresponding to the sequence −1512 to +6 (the start codon ATG of human WIF-1 gene is defined as +1). FailSafe PCR (Epicentre, Madison, Wisconsin) (38 cycles) was performed using human genomic DNA isolated from cultured cells and normal tissue as template. To amplify the different fragments we used the following primers: construct 1, forward—5′-GCCAGCTTTGCCA GTCTTAC-3′, reverse—5′-TGCTCAGGACCTCCTCGCTG-3′ (from −292 to +6); construct 2, forward—5′-GAGTGATGTCCCAGGGG TCT-3′, reverse—5′-GTAAGACTGGCAAAGCTGGC-3′ (from –557 to –272); construct 3, forward—5′-GGAGGGTCAGGTACAGCT AT-3′, reverse—5′-AGACCCCTGGGACATCACTC-3′ (from –1012 to –535); construct 4, forward—5′-GCTGGGCCAGGTGGA TAGGT-3′, reverse—5′-AGACCCCTGGGACATCACTC-3′ (from −1512 to −535); and construct 5, forward—5′-GCTGGGCCAGG TGGATAGGT-3′, reverse—5′-TGCTCAGGACCTCCTCGCTG-3′ (from −1512 to +6). The position of all the primers is also marked in Fig. 1. The PCR products were extracted from agarose gel using an extraction kit (QUIAquick Gel Extraction kit, Qiagen, Valencia, CA) and were subsequently sequenced at the DNA-sequencing Core Facility of the University of California, San Francisco Comprehensive Cancer Center.

Construction of the human WIF-1 promoter region. We used PCR to generate truncated forms of the human WIF-1 promoter region (−1512 to+6) and subcloned them into the *Nhe*I (5′) and *Hin*dIII (3′) sites of pGL3Basic vector purchased from Promega (Madison, WI). Sense primers for generating truncated forms described above contain an adaptor with an *Nhe*I restriction site (5′-CCAATTGCTAGC-3′) at the 5′ end. Anti-sense primers contain an adaptor with a *Hin*dIII restriction site (5′-CCTTGGAAGCTT-3′) at the 5′ end. Then pGL3Basic vector and all PCR products that have *Nhe*I site at 5′ and

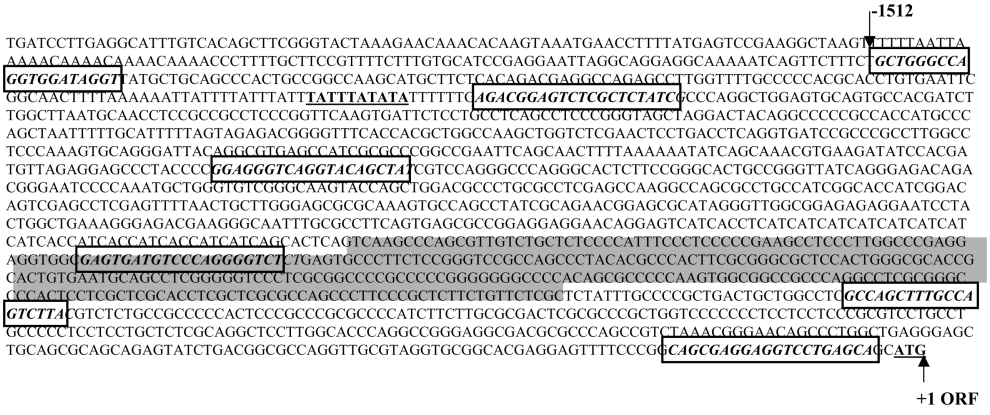

Fig. 1. The 5′ flanking region of human WIF-1 gene. A ∼1.5 kb fragment was amplified using genomic DNA as a template. A standard PCR was used to amplify the different fragments. Sequences corresponding to the primers used for the amplification are in bold and boxed. Putative ATG open reading frame codon side (defined as +1) as well as the TATA box-containing region is in bold and underlined. The gray box indicates a rich CpG island region found within the upstream promoter region. The 5′ end of the region cloned is indicated as −1512. The sequence cloned includes the whole sequence from +1 to −1512. The sequence was obtained from the human genome sequence database at the UCSF web server (http://genome.ucsf.edu/) and the promoter search program (http://www.genomatix.de/).

*Hin*dIII site at 3′ were doubly digested with *Nhe*I and *Hin*dIII enzymes (New England Biolabs, Beverly, MA). The digested inserts were gel-purified by electrophoresis and ligated (Quick Ligation Kit, New England Biolabs, Beverly, MA) to generate the deletion constructs. The constructs were then transformed into competent *Escherichia coli* (One Shot Competent E.Coli, Invitrogen, Carlsbad, CA) for amplification. The created plasmids were purified by using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and afterwards confirmed by restriction enzyme digestion. All constructs were sequenced using GLprimer2 (counter clockwise) and RVprimer3 (clockwise) to check the correct orientation of the insert inside the vector (primers purchased from Promega, Madison, WI).

Transfection and promoter activity analysis. One day before transfection, cells (2×10^5) were plated in six-well plates with growth medium without antibiotics. When cells reached 80–90% confluence they were co-transfected using 2.0 μg of each DNA construct in pGL3Basic vector and 0.05 μg pRL-TK Vector (Promega, Madison, WI) containing *Renilla* luciferase as an internal control for the transfection efficiency. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to mediate transfection. The resulting cells were cultured in the medium for an additional day and subjected to luciferase assay. One day after the transfection, cells were lysed in a lysis buffer and firefly and *Renilla* luciferase activities in cells of each well were measured using Dual-Luciferase Assay System (Promega, Madison, WI) and a luminometer. All the measured luciferase activities were normalized to pRL-TK Vector activity and were given relative to the basal activity of empty pGL3Basic vector, which was set to unity. The data shown represent mean values (±SD). All measurements were performed in triplicate and repeated in at least three independent experiments.

Western blotting. Standard protocol was used. Whole cell lysates (A549, SW480, HCT116, H28, and MS-1) were obtained with M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). Cytosolic fraction was prepared according to a protocol described previously [24]. Anti-β-catenin mouse monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-β-actin mouse monoclonal antibody was obtained from Sigma (St. Louis, MO).

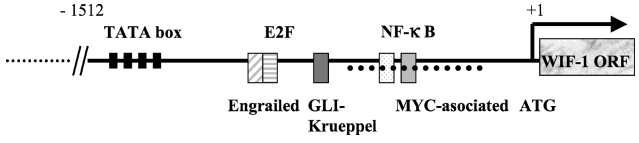

Fig. 2. Schematic representation of human WIF promoter region. A ~1.5 kb segment is shown. Binding sites of transcription factors such as Engrailed, E2F, GLI-Krueppel, NF-κB, and MYC are indicated as well as the rich CpG island region (dot lines) and TATA box.

Statistical analysis. Unpaired *t* test was used for comparing activities of different constructs.

Results

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 UCSC web server (http://genome.ucsc.edu/). We used a promoter search program (http://www.genomatix.de/) to confirm that the 5′ flanking region of the gene presents classical features of a promoter region (Fig. 1). Several important transcription sites were observed in this fragment such as Homobox Protein Engrailed (−826 to −830 from ATG), E2F (−807 to −811), GLI-Krueppel (−548 to −552), MYC-associated (−419 to −423), and NF-κB (−421 to −425) (Fig. 2). We observed that the G/C content of the human WIF-1 promoter region is high (approximately 63.5%). We then used a CpG island search program (http://www.uscnorris.com/cpgislands/cpg.cgi) to map the CpG dinucleotides within the WIF-1 promoter and found a CpG island (105 CpGs) in this promoter (Figs. 1 and 2). In addition, we found that this promoter region contained one putative TATA box (−1368 to −1378 ahead of the ATG) (Figs. 1 and 2). We also found that the human WIF-1 promoter region is largely conserved compared to the chimp promoter (99% identity), but less conserved in mouse and rat promoters (approximately 5.9% and 5.7%, respectively) (Fig. 3).

Functional analysis of the human WIF-1 promoter constructs

We cloned the complete 1506-bp fragment (from the TATA box to the ATG), as well as five truncated fragments into a promoterless luciferase (LUC) expression vector pGL3Basic (Fig. 4A). These constructs were transfected into 293T cells and LUC activities were then

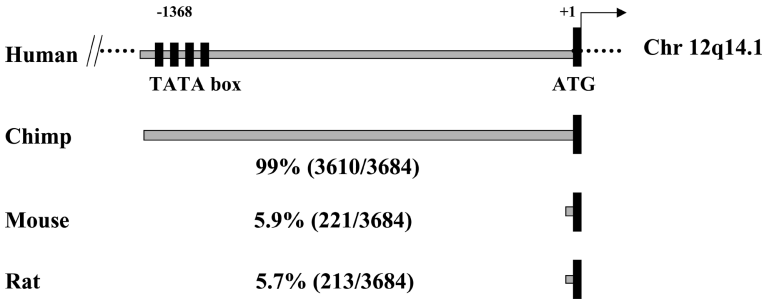

Fig. 3. Comparison of WIF-1 promoter regions of human, chimp, mouse, and rat. The identical region corresponds to the 5′ site before the ATG of open reading frame. In parentheses, the number of identical amino acids in those regions is shown.

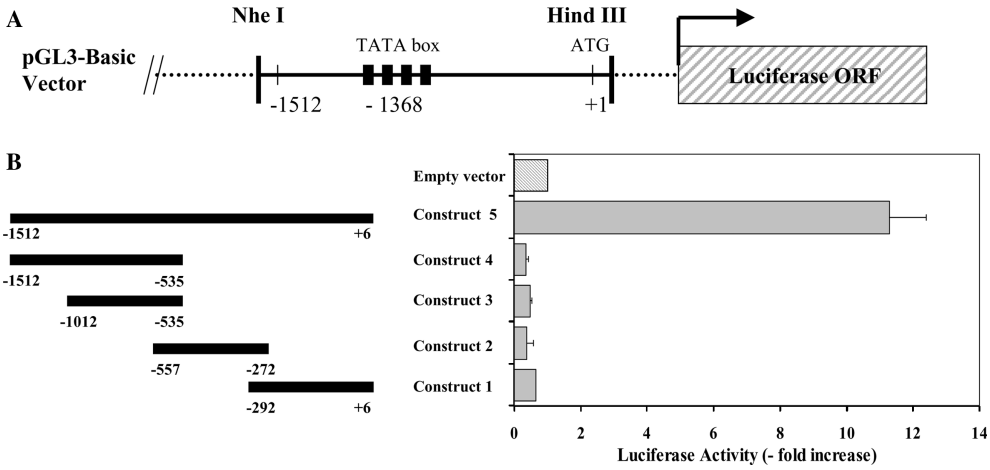

Fig. 4. (A) Schematic representation of the ~1.5 kb construct used in this experiment inserted in a pGL3Basic vector (pGL3B) upstream of the firefly luciferase gene. The restriction sites used to clone the different constructs (*Nhe*I/*Hin*dIII) are shown. (B) Luciferase activity of different constructs of the genomic human WIF-1 promoter. The constructs correspond to 5 different segments of a ~1.5 kb region of the 5′ promoter region. They were inserted into pGL3Basic vector upstream of firefly luciferase gene (described in Materials and methods). Each promoter construct was verified by sequencing. 293T cells were transiently transfected with each construct. All relative luciferase activities were normalized to the activity of an empty pGL3Basic vector (pGL3B) and pRL-K was used as an internal control. Cells were harvested 24–36 h after transfection, and cell lysates were assayed for luciferase activity in a luminometer using Dual-Luciferase Assay Reporter System. Results are expressed as the ratio of activity of firefly to *Renilla* luciferase. Results are means ± SD (error bars) of at least three individual experiments.

measured (Fig. 4B). The four truncate constructs with deletion of some part of the whole region (construct 1; −292/+6 construct 2; −557/−272, construct 3; −1012/−535, and construct 4; −1512/−535) did not display any basal activity. Basal activity of the construct that contained all the elements (construct 5: 1512/+6) was significantly higher (approximately 11.3-fold increase of LUC activity) than those described above (construct 1, 2, 3, and 4) ($p < 0.02$). Taken together, these results indicate that the integrity of the WIF-1 promoter is likely necessary for its activity. Therefore, our results confirm that the whole fragment that we amplified (−1512/+6) could function as a promoter element.

Analysis of the WIF-1 promoter activity in human cancer cell lines

Next, we transfected the complete wild type WIF-1 promoter construct (construct 5) into different human cancer cell lines, including a non-small-cell lung cancer cell line (A549), mesothelioma cell lines (H28, MS-1), and colon cancer cell lines (HCT116, SW480) and examined the LUC activity after 24–48 h (Fig. 5A). We found that this construct (construct 5) displayed significantly high LUC activity in A549, SW480, and HCT116 cell lines (7- to 8-fold increase compared to that of empty vector) ($p < 0.04$), but only moderate LUC activity in the two mesothelioma cell lines MS-1 and H28. Interestingly, we found that the WIF-1 promoter activity correlates with the level of cytosolic β-catenin expression in these cell lines (Fig. 5B). These results suggest that the WIF-1 promoter may be under the regulation of the Wnt/β-catenin pathway.

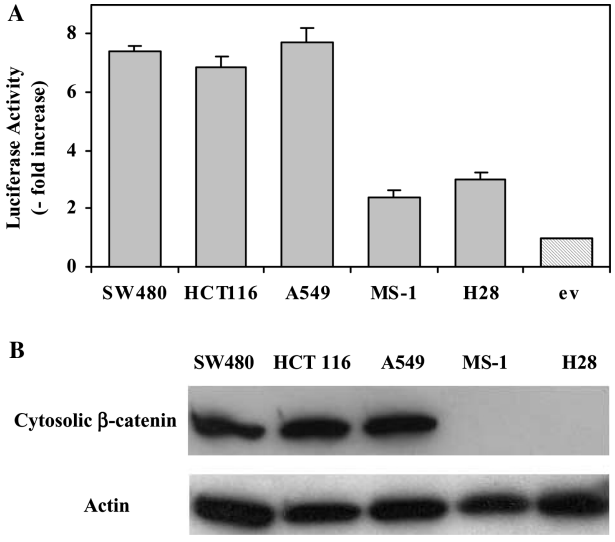

Fig. 5. (A) Luciferase activity of the complete human ~1.5 kb WIF-1 promoter construct in a pGL3Basic vector transfected in NSCLC cell line (A549), mesothelioma cell lines (H28, MS-1), and colorectal carcinoma cell lines (HCT 116, SW480) The average means ± SD (errors bars) are shown of at least three individual experiments. (B) Western blot analysis of cytosolic β-catenin in the different cell lines (A549, H28, MS-1, HCT 116, and SW480). β-Actin was used as loading control.

Discussion

The Wnt pathway plays a significant role in carcinogenesis. It is well known that mutations in the adenomatous polyposis coli gene (APC), axin, or β-catenin lead to β-catenin accumulation in the nucleus which is often observed in human cancers [25]. Moreover, β-cate-

nin does not bind to DNA itself, but needs to bind to TCF/LEF transcription factors for transactivation in the nucleus of a specific subset of genes [26,27]. Although the intracytoplasmic Wnt pathway has been widely studied, little is known about the relation between the antagonists of Wnt ligands and their role in carcinogenesis. Recently, however, information about the role of these secreted proteins in carcinogenesis has been elucidated. For instance, secreted frizzled-related proteins (sFRPs), one of the secreted Wnt protein inhibitors, are transcriptionally downregulated in mesothelioma [28] and in colorectal carcinoma [17]. In both tumors sFRPs promoter hypermethylation was identified. Furthermore, we recently found that WIF-1 transcription is silenced in human lung cancer and that this silencing is due to frequent hypermethylation [22].

In this study, we cloned and characterized approximately ~1.5 kb of the 5′ genomic region of the human WIF-1 gene. This 5′ region shows baseline promoter activity and contains one putative TATA sequence and multiple potential transcription factor binding sites. The whole human construct 5 (−1512 to +6) that we cloned showed significantly high luciferase activity (~11.3-fold increase). Taken together, these results suggest that we have found a functional human WIF-1 promoter. Next, we examined promoter activity of the different truncated 5′ constructs (construct 4, −1512 to −535; construct 3, −1012 to −535; construct 2, −557 to −272; and construct 1, −292 to +6). When we used these truncated constructs we found that promoter activity dramatically decreased ($p < 0.02$). These results indicate that the entire WIF-1 promoter region may be needed for its activity.

These results are consistent with our recent data [22]. The functional WIF-1 promoter region that we analyzed contains the CpG island that we previously described as an important issue in Wnt signaling activation. It is well known that aberrant methylation of CpG islands is one of the major modes of inactivation of tumor suppressor genes in cancer and a growing list of genes is being identified as having abnormal methylation of promoters having CpG islands [29]. Taken together, our results demonstrate that the CpG island lies inside a functional WIF-1 promoter, thus holding this methylated region as an important mechanism of the WIF-1 gene silencing and therefore constitutive activation of Wnt/β-catenin pathway.

The human WIF-1 promoter construct that we cloned allowed us to examine the transcriptional activity of WIF-1 in different human cancer cell lines. We found that WIF-1 promoter activity of the whole fragment was different in the cell lines that we tested. Cell lines that lack cytosolic β-catenin expression (MS-1, H28) revealed lower LUC activity than those with high expression level of cytosolic β-catenin (SW480, HCT116, and A549), suggesting a correlation between expression levels of cytosolic β-catenin and WIF-1 promoter activity. It is known that Wnt pathway antagonists such as sFRP [30–32], Cerberus [33], and WIF-1 [19] prevent Wnt ligands from binding to their receptors and thereby inhibit the Wnt/β-catenin pathway. Little was known, however, about the regulation mechanisms of these Wnt pathway inhibitors. Our findings argue that WIF-1 could be a downstream target gene of Wnt/β-catenin pathway and may function as a feedback inhibitor of Wnt signaling. The two cell lines lacking cytosolic β-catenin (MS-1 and H28), however, still have some activity when compared with the empty vector. These data could be explained by the fact that Wnt/β-catenin pathway may not be the only pathway that controls WIF-1 expression although Wnt/β-catenin could be the major pathway of WIF-1 activation.

In summary, our study provides more insight into the regulation of WIF-1 expression and opens a way to future investigations on the role of WIF-1 in Wnt/β-catenin activation during carcinogenesis.

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