Wnt inhibitory factor inhibits lung cancer cell growth

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Objective: Aberrant activation of the Wnt signaling pathway is associated with the pathogenesis of multiple cancers, including non-small cell lung cancer. Wnt inhibitory factor, a secreted Wnt antagonist, is downregulated in non-small cell lung cancer. We hypothesized that restoration of Wnt inhibitory factor function would inhibit lung cancer cell growth.

Methods: The lung cancer cell lines A549 and H460 were transfected with an expression vector containing the Wnt inhibitory factor gene. Apoptosis rates and colony formation were measured after transfection. Recombinant Wnt inhibitory factor protein was used to treat H460 cells, and proliferation rates were measured with an MTS assay. Finally, Wnt inhibitory factor plasmid was peritumorally injected near H460 tumor xenografts in nude mice.

Results: Wnt inhibitory factor-transfected cells had increased apoptosis and decreased colony formation than control cells. Recombinant human Wnt inhibitory factor protein was also able to inhibit H460 cell proliferation measured by using the MTS assay. Wnt inhibitory factor plasmid significantly inhibited the growth in vivo of H460 tumor xenografts in nude mice.

Conclusion: These data suggest that Wnt inhibitory factor is able to inhibit lung cancer cell growth both in vitro and in vivo and provides additional evidence that Wnt inhibitory factor plays an important role in Wnt pathway regulation in lung cancer.

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As the leading cause of cancer death worldwide, lung cancer poses a major public health problem. Non-small cell lung cancer (NSCLC) comprises 75% to 80% of lung cancer cases.¹ Despite recent advances in treatment, overall prognosis remains poor, and the overall 5-year survival rate remains at 15%.² A better understanding of the molecular pathogenesis of lung cancer might lead to more effective targeted therapies.

The Wnt signaling pathway is a key developmental pathway that has also been implicated in oncogenesis.^{3,4} It plays an important role in normal embryonic and stem cell development but is dormant in most adult tissues. Aberrant reactivation of the Wnt pathway has been shown in multiple human cancers, including NSCLC.⁵⁻¹⁰

The Wnt pathway is regulated by the complex interplay of multiple agonists and antagonists. One such antagonist is Wnt inhibitory factor (WIF-1).

Abbreviations and Acronyms

RT-PCR = reverse transcription–polymerase chain reaction

WIF-1 = Wnt inhibitory factor

WIF-1 is a naturally occurring secreted protein that binds the Wnt ligand.¹¹ Downregulation of WIF-1 has been shown in multiple cancers.^{12–14} We recently reported that WIF-1 was downregulated and silenced by promoter hypermethylation in NSCLC.¹⁵

We hypothesized that restoration of WIF-1 function would inhibit the Wnt pathway and thus inhibit lung cancer cell growth.

Materials and Methods**Cell Lines**

NSCLC cell lines (NCI-H460 and A549) were obtained from American Type Culture Collections (Manassas, Va). NSCLC cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL).

Transfection

The human *WIF-1* open reading frame was cloned into a mammalian expression vector, pcDNA3.1, obtained from Invitrogen (Carlsbad, Calif). Twenty-four hours before transfection, 2×10^5 cells (H460 or A549) were plated in 6-well plates. Eight micrograms of *WIF-1* plasmid was transfected per well by using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's protocol. Eight micrograms of pcDNA3.1 vector containing the *lac-Z* gene (Invitrogen) was transfected into cells as a control.

Semiquantative Reverse Transcription–Polymerase Chain Reaction

Seventy-two hours after transfection, H460 and A549 cells were treated with trypsin and harvested. Total RNA from was isolated with an RNeasy Mini Kit (Qiagen, Valencia, Calif), according to the manufacturer's protocol. Reverse transcription–polymerase chain reaction (RT-PCR) was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif) by using an RT-PCR kit (SuperScript One-step RT-PCR with Platinum Taq kit, Invitrogen) for 25 cycles, according to the manufacturer's protocol. Primers for RT-PCR were obtained from Operon Technologies, Inc (Alameda, Calif). Primer sequences for the human *WIF-1* cDNA were 5'-CCGAAATGGAGGCTTTTGTA-3' (forward) and 5'-TGGTTGAGCAGTTTGCTTTG-3' (reverse), primers for *c-MYC* were 5'-TTCGGGTAGTGGAACACAG-3' (forward) and 5'-CAGCAGCTCGAATTTCTTCC-3' (reverse), and primers for *cyclin D1* were 5'-CTGTCGCTGGAGCCCGTGAA-3' (forward) and 5'-TGGCACAGACCCGAACGAAG-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. RT-PCR products were then electrophoresed on an agarose gel and photographed under UV light.

Apoptosis Assay

Three days after transfection, cells were harvested after treatment with trypsin. They were then stained with an Annexin V FITC Apoptosis Detection Kit (Invitrogen Biosource, Carlsbad, Calif), according to the manufacturer's protocol. Then stained cells were immediately analyzed by means of flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ).

Colony Formation Assay

Transfection was performed as above. Twenty-four hours after transfection, cells were stripped and plated on 6-well cell-culture dishes. The cells were then selected by using G418 (400 µg/mL). Two weeks after selection, colonies were stained with 0.5% crystal violet solution and counted.

Recombinant WIF-1 and Cell Proliferation Assay

Purified recombinant human WIF-1 protein was obtained from R&D Systems (Minneapolis, Minn). The protein was resuspended in phosphate-buffered saline to obtain concentrations of 1 µg/µL.

H460 cells were plated at 5×10^3 cells per well in RPMI medium in a 96-well culture plate. Twenty-four hours after plating, half the cells were treated with recombinant WIF-1 protein, and half were treated with bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, Mo) to achieve a concentration of 20 µg/mL. Medium was changed after 96 hours, at which point WIF-1 or BSA was again added to achieve concentrations of 20 µg/mL. The percentage of live cells was evaluated by incubating for 2 hours with the Celltiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega, Madison, Wis). Plates were read with a spectrophotometer at a wavelength of 490 nm. Measurements were made at 3 time intervals: 24 hours after treatment, 96 hours after treatment, and 192 hours after treatment. Three wells from each treatment group were measured for each time point.

Tumor Xenografts

All in vivo experiments were performed in accordance with the University of California, San Francisco institutional guidelines (institutional review board A8714-25971-01). Groups of 8 female athymic nude mice (strain NCRNU-M, 5–6 weeks old) received subcutaneous injections with 1×10^7 H460 cells in the dorsal area at a volume of 100 µL. Three days later, the tumors were uniformly formed. We then subcutaneously injected the mice twice weekly in the peritumoral area with either 50 µg of *WIF-1* vector or empty pcDNA3.1 vector in 100 µL of lipofectamine. Tumor size was determined at weekly intervals for 3 weeks, and tumor volume was calculated by using width (x) and length (y; $[x^2y/2]$, where x if less than y). After the mice were killed, tumors were resected and weighed.

Statistical Analysis

The data shown represent mean values $\pm$ standard deviation. The unpaired *t* test was used to compare different treatments and determine the *P* value.

Results

Previously, we found that WIF-1 was underexpressed in the NSCLC cell lines H460 and A549.¹⁵ An expression vector

Figure 1. Reverse transcription–polymerase chain reaction for Wnt inhibitory factor (*WIF-1*) in A549 and H460 cells transfected with either *lac-Z* (control) or *WIF-1*. The fragment of human *WIF-1* cDNA amplified is 188 bp.

containing the *WIF-1* gene was transfected into H460 and A549 cells to test whether *WIF-1* can inhibit growth and induce apoptosis in these cell lines. Vector containing *lac-Z* was used as a control. We confirmed increased *WIF-1* expression in the *WIF-1*–treated cells by performing semi-quantitative RT-PCR on RNA extracted from *WIF-1*– and *lac-Z*–transfected cells (Figure 1).

We confirmed downregulation of the Wnt pathway by measuring expression of the Wnt target genes *c-MYC* and *cyclin D1* by using semi-quantitative RT-PCR. We found that lung cancer cells transfected with *WIF-1* had decreased expression of *c-myc* and *cyclin D1* (Figure 2). Transcription of both signals has been shown to be mediated by β -catenin through the TCF binding site.¹⁶⁻¹⁸

Three days after transfection, we found significantly increased apoptotic cell death in cells transfected with *WIF-1* ($P < .05$, Figure 3). Having found increased apoptosis in *WIF-1*–transfected cells, we then assessed the effect of *WIF-1* on growth inhibition. We selected for transfected cells and found that after selection, cells transfected with *WIF-1* formed dramatically fewer colonies than did cells transfected with control vector (Figure 4).

We then used recombinant human *WIF-1* to validate the effect of *WIF-1* in NSCLC cells. Purified recombinant human *WIF-1* protein was added to the medium of H460 cells

Figure 2. Reverse transcription–polymerase chain reaction for *cyclin D1* and *c-myc* in A549 and H460 cells transfected with either *lac-Z* (control) or Wnt inhibitory factor (*WIF-1*). Glyceraldehyde-3-phosphate dehydrogenase was used as a control transcript.

Figure 3. Annexin V analysis of apoptosis. Seventy-two hours after transfection with either *lac-Z* (control) or Wnt inhibitory factor (*WIF-1*), A549 and H460 cells were harvested by means of trypsinization and stained with an Annexin V–fluorescein isothiocyanate kit. The bar graphs show the percentage of apoptosis, which represents the number of Annexin V⁺ cells out of the total number of cells counted (mean $\pm$ standard deviation, $P < .05$).

plated in 96-well plates. We used an MTS assay to assess cell proliferation at 24 hours, 96 hours, and 192 hours after treatment. We found that recombinant human *WIF-1* significantly inhibited cell growth after 8 days of incubation (Figure 5).

Finally, we assessed the effect of *WIF-1* transfection in an in vivo tumor xenograft model. Nude mice were subcutaneously injected with H460 cells and then treated with peritumoral injections of *WIF-1* plasmid or empty vector. We found the *WIF-1* plasmid significantly inhibited tumor growth (Figure 6, A). After 4 weeks of treatment, the tumors treated with the control weighed more than 3 times as much as tumors treated with *WIF-1* ($P < .05$; Figure 6, B). There

Figure 4. Colony formation assay with A549 and H460 cells. The cells were transfected with *lac-Z* or Wnt inhibitory factor (*WIF-1*) vector and selected with G418. Colonies were stained by using 0.5% methylene blue and counted 2 weeks after the transfection.

Figure 5. MTS assay of H460 cells treated with recombinant Wnt inhibitory factor (*WIF-1*) or BSA for 8 days. **A**, Proliferation was measured with the MTS assay at 3 time points: 1 day, 4 days, and 8 days after treatment. Absorbance was measured at 490 nm 2 hours after addition of MTS reagent. **B**, MTS assay after 8 days of treatment. Absorbance at 490 nm is expressed as percentage proliferation, where control treatment (BSA) is 100% (mean $\pm$ standard deviation, $P < .05$).

was no statistically significant difference in the overall weight of the mice, nor did there appear to be any obvious toxicity from the *WIF-1* treatment.

Discussion

We found that restoration of *WIF-1* function caused Wnt pathway downregulation and inhibited NSCLC cell growth. Transfection with *WIF-1* induced apoptosis in NSCLC cells and inhibited colony formation. Transfection also inhibited NSCLC tumor xenograft growth. Treatment with recombinant *WIF-1* protein also inhibited NSCLC proliferation. These results support our previous studies, which showed that inhibition of Wnt signaling could inhibit NSCLC cell growth.¹⁹ The results also reinforce the hypothesis that downregulation of *WIF-1* is important for Wnt pathway activation in NSCLC.

Aberrant activation of the Wnt pathway has recently been linked to lung cancer. Traditional alterations in the Wnt pathway, such as β -catenin and adenomatous polyposis coli gene mutations are relatively uncommon in lung cancer.²⁰⁻²² However, there is growing evidence that other mechanisms of Wnt pathway activation do play an important role in lung cancer. We have found evidence of Wnt pathway activation in lung cancer mediated through the molecule disheveled.⁷ In addition, multiple studies have shown downregulation of secreted Wnt inhibitors in lung cancer.^{15,23-25} *WIF-1* is among these secreted Wnt inhibitors and is known to bind Wnt proteins and inhibit their activities.²⁶ The mechanism of interaction between *WIF-1* and Wnt remains poorly understood. Unlike the multiple

Figure 6. Wnt inhibitory factor (*WIF-1*) plasmid treatment in an in vivo model using H460 tumor xenografts subcutaneously implanted onto nude mice. Mice underwent peritumoral injections of empty vector or *WIF-1*. **A**, Measurements were made at bi-weekly intervals to determine tumor volume. **B**, After the mice were killed, tumors were resected and weighed (mean $\pm$ standard deviation, $P < .05$).

secreted, frizzled related proteins that have been described, *WIF-1* lacks any sequence similarity with the cysteine-rich domain of the Wnt target frizzled receptor.²⁷ Nonetheless, *WIF-1* does bind to Wnt proteins in the extracellular space and inhibits Wnt-frizzled interactions.¹¹ The relative importance of *WIF-1* among the various Wnt antagonists is also unclear.

We have previously shown that *WIF-1* is downregulated in the vast majority of NSCLC tumor specimens.¹⁵ The ability of *WIF-1* to inhibit NSCLC growth in vitro and in vivo thus has potential therapeutic implications. These experiments reinforce the potential of using demethylating agents to reactivate hypermethylated tumor suppressors in cancer and raise the possibility of therapy specifically related to the role of *WIF-1* in the Wnt pathway.²⁸

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Discussion

Dr Y. Joseph Woo (Philadelphia, Pa). Does the WIF block the canonical and noncanonical Wnts or one group in particular?

Dr Kim. That is a good question. On the basis of our Western analysis, it seems that there is some inhibition of the canonical pathway because there is some decrease in β -catenin levels. We have looked at disheveled levels as a marker of noncanonical pathways, and our results thus far have been mixed. It seems that in some of the cell lines we have tested, disheveled levels are decreased, but in others they stay the same.

Dr Robert J. Cerfolio (Birmingham, Ala). Excellent work. Congratulations. Now, please take me on your Disney World ride of the future 3 years, 5 years, 7 years from now. Tell me how you envision your data helping us treat patients in the land of tomorrow. Is it a targeted therapy with radiotherapy, is it a targeted therapy with adjuvant chemotherapy, neoadjuvant therapy? How do you see this affecting patient care 10 years from now?

Dr Kim. That is a great question. I think WIF is a good target for therapy because it acts in the extracellular space. Therefore a lot of problems of intracellular delivery can be avoided. However, what I did not show you was that the protein concentrations that we required to actually have an effect were really quite high. Therefore I do not think that recombinant WIF by itself could be used as a therapy. We have some other experiments that seem to indicate there is some kind of an autocrine effect, as well as a paracrine effect. I think that future avenues could be something along the lines of a fusion protein or some type of protein that has the active moiety along with some other delivery molecule.

Dr Mark J. Krasna (Baltimore, Md). Dr Kim, Dr Byrne and I are curious. What does Wnt stand for, or after whom is it named?

Dr Kim. As I alluded to, the Wnt pathway is a key pathway in development, and it is named after a combination of the wingless gene of *Drosophila* because mutations lead to wing malformation and the *Int* gene in mice, the integration site for the mouse mammary tumor virus.