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Frizzled-8 as a putative therapeutic target in human lung cancer

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ABSTRACT

Lung cancer is the leading cause of cancer related deaths worldwide. It is necessary to better understand the molecular mechanisms involved in lung cancer in order to develop more effective therapeutics for the treatment of this disease. Recent reports have shown that Wnt signaling pathway is important in a number of cancer types including lung cancer. However, the role of Frizzled-8 (Fzd-8), one of the Frizzled family of receptors for the Wnt ligands, in lung cancer still remains to be elucidated. Here in this study we showed that Fzd-8 was over-expressed in human lung cancer tissue samples and cell lines. To investigate the functional importance of the Fzd-8 over-expression in lung cancer, we used shRNA to knock down Fzd-8 mRNA in lung cancer cells expressing the gene. We observed that Fzd-8 shRNA inhibited cell proliferation along with decreased activity of Wnt pathway in vitro, and also significantly suppressed A549 xenograft model in vivo ($p < 0.05$). Furthermore, we found that knocking down Fzd-8 by shRNA sensitized the lung cancer cells to chemotherapy Taxotere. These data suggest that Fzd-8 is a putative therapeutic target for human lung cancer and over-expression of Fzd-8 may be important for aberrant Wnt activation in lung cancer.

1. Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) is more common and represents approximately 80–85% of all lung cancers [1]. Current treatment strategies for lung cancer include surgical resection, chemotherapy, radiation therapy, targeted therapy or a combination of treatments depending on the type of lung cancer and its stage level. Despite advances made in these treatments, lung cancer remains highly lethal with a 5-year survival rate of less than 15% [2]. Therefore, new effective therapies for lung cancer are desperately needed. An increased understanding of the molecular mechanisms underlying lung cancer development and progression should lead to the design of better targeted therapies in the treatment of this deadly disease.

Mammalian Wnt proteins comprise a family of nineteen highly conserved and secreted glycoproteins. Secreted Wnt ligands have been shown to activate signal transduction pathways and trigger changes in gene expression, cell behavior, adhesion and polarity [3]. Receptors for the Wnt proteins are the Frizzled (Fzd) family of receptors. Transduction of Wnt signaling begins with Wnt ligands binding to the cysteine-rich domain (CRD) of the Fzd receptors at the cell membrane initiating either the 'canonical' or 'non-canonical' pathway [3]. In the canonical Wnt pathway, Wnt binds to the Fzd receptors, activates Dishevelled (Dvl) and disassembles the β-catenin "destruction complex," which prevents the phosphorylation and subsequent ubiquitination of β-catenin, resulting in β-catenin stabilization and accumulation in the cytoplasm. Stabilized β-catenin enters the nucleus, where it complexes with TCF/LEF transcription factors to regulate the transcription of downstream target genes [3].

Aberrant activation of the canonical Wnt signaling pathway is associated with a variety of human cancers, including thoracic malignancies. For example, both Wnt-1 and Wnt-2 are up-regulated in NSCLC [4,5], while Wnt-7a is down-regulated in most lung cancer cell lines and tissues. Co-expression of Wnt-7a and Fzd-9 has been shown to inhibit cell growth of NCSLC cell lines [6]. Dishevelled (Dvl) is over-expressed in 75% of microdissected NSCLC tissues [7]. In addition, methylation-silencing of secreted Wnt antagonists: Wnt Inhibitory Factor-1 (WIF-1) and secreted Frizzled-related proteins (sFRPs) has been reported to be associated with aberrant Wnt activation in lung cancer [8–10]. Mutations in key Wnt signaling genes such as APC or β-catenin, frequently associated with colon cancer, seem to be rare in lung cancer [3]. Thus, Wnt pathway may be activated upstream of β-catenin [11–13]. However, the role that the Fzd receptors may play in

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the pathogenesis of lung cancer still remains to be elucidated. In this study, we sought to investigate whether Fzd-8 plays an important role in aberrant activation of Wnt signaling pathway in human lung cancer. We examined Fzd-8 expression in fresh human lung cancer tissue specimens and cell lines, as well as relationship between Fzd-8 function and the canonical Wnt pathway in lung cancer cells.

2. Materials and methods

2.1. Cell lines and tissue samples

NSCLC cell lines were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured in RPMI 1640 medium. All cell cultures 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.

Fresh lung cancer and adjacent normal lung tissues from patients were collected at the time of surgical resection and immediately snap-frozen in liquid nitrogen at Tianjin Cancer Hospital and Tianjin Chest Hospital. These tissue samples were kept at −80 °C before use.

2.2. RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted from lung cancer cell lines and tissues using the TRIzol reagent (Tiangen) according to the manufacturer's protocol. RT-PCR was performed as follows: cDNA was produced using AMV reverse transcriptase (Promega) and N9 random primers; then PCR was done in GeneAmp 2700 (Applied Biosystems) using the cDNA as template. Taq enzyme and PCR reagents were purchased from Tiangen Corp. Primers were purchased from Sangon Corp. Primer sequences used for human Fzd-8 were: 5′-GGACTACAACCGCACCGACCT-3′ (forward) and 5′-ACCACAGGCCGATCCAGAAGAC-3′ (reverse). The housekeeping gene β-actin was amplified as an internal control. PCR condition: 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s for 35 cycles, followed by a final extension at 72 °C for 10 min. Semi-quantitative RT-PCR products in cell lines and tissue samples were analyzed on 1% agarose gel electrophoresis and stained with ethidium bromide.

2.3. Western blotting

Cytosolic proteins were prepared as follows: cell pellet was suspended in hypotonic buffer (20 mM Tris–Cl, pH 7.5, 25 mM NaF and 1 mM EDTA) and set on ice for 30 min. It was spun down at 45,000 rpm in an ultracentrifuge (Optima MAX, Beckman Coulter) at 4 °C for 30 min. The supernatant was then collected as cytosolic proteins. Proteins were separated on 4–15% gradient SDS–polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore). Primary antibodies used were anti-β-actin (1:5000; Sigma–Aldrich) and anti-β-catenin (1:2000; Sigma–Aldrich). Antigen–antibody complexes were detected by ECL blotting analysis system (Amersham Pharmacia Biotech).

2.4. Transfection and RNA interference

Two Fzd-8 shRNA (their targeted human Fzd-8 sequences are: (Fzd-8 shRNA-1) 5′-AAGACAGGCCAGATCGCTAAC-3′ and (Fzd-8 shRNA-2) 5′-CTGTGCATGGACTACAACCGC-3′, respectively), and control (non-silencing) shRNA (in pRFP-C-RS vector) were purchased from Origene. Cell lines were plated in six-well plates with fresh media without antibiotics for 24 h before transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were re-plated in 10 cm dishes for selection with G418 (500 μg/ml; Invitrogen). Stable transfectants were maintained in regular medium with G418 (300 μg/ml) for further analysis.

2.5. Cell proliferation assay

The growth rates were determined by CellTiter 96 Aqueous Non-Radiotive Cell Proliferation Assay Kit (Promega). The stable cell lines were plated into 96-well tissue culture plates with the number of 5×10^2 cells/well. The MTS solutions were added to the medium at different time points and incubated for 1.5 h. The absorbance at 490 nm was measured using a microplate reader (model 680; Bio-Rad).

2.6. Colony formation assay

Five hundred individual cells of the stable lines were seeded in 10 cm dishes and cultured for 2 weeks. Colonies were then fixed by 10% formalin, stained with 0.5% crystal violet and counted.

2.7. Xenograft model

The mice experiments were conducted in the animal facility of Tianjin Medical University Cancer Institute and approved by the Institutional Animal Care and Use Committee. Lung cancer xenografts were established with 6-week-old female BALB/c nude mice. Briefly, A549 cells stably transfected with either control or Fzd-8 shRNA were trypisnized and resuspended in PBS (pH 7.4). The cell suspensions were then mixed with matrigel (Vigorous) (volume ratio: 1:1) at 4 °C. The mixture containing 5×10^6 cells in a volume of 100 μl was s.c. injected into the flanks of female mice (5 mice/group). Tumor size was determined by multiplying by $0.5 \times \text{width}^2 \times \text{length}$.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows. The values represent the means ± standard deviations (S.D.) (error bars) of triplicate of three independent experiments. The difference between groups was determined by Student's *t*-test and a p value of $\leqslant 0.05$ was considered significant.

3. Results

3.1. Over-expression of Fzd-8 in primary lung cancer tissue samples and cell lines

We first used semi-quantitative RT-PCR to examine Fzd-8 mRNA expression in human primary lung cancer tissue samples (Fig. 1A). In 20 tissue samples from lung cancer patients examined, we observed up-regulation of Fzd-8 expression in 85% (17 of 20) tumor samples when compared to their matched normal lung tissues. In the cases nos. 15 and 16, the Fzd-8 expression was not detectable in both cancerous and their matched normal tissue samples. We also used semi-quantitative RT-PCR to examine Fzd-8 mRNA expression in human lung cancer cell lines (Fig. 1B). We found that the Fzd-8 gene was highly expressed in 5 of 6 lung cancer cell lines (A549, H1299, H1650, A427, and H322) that we examined, consistent with our observation in lung cancer tissue samples. Because aberrant over-expression of Wnt ligands has been reported in human lung cancer [5,14], our findings suggest that there may be a functional significance of the Fzd-8 over-expression in aberrant activation of Wnt signaling in human lung cancer.

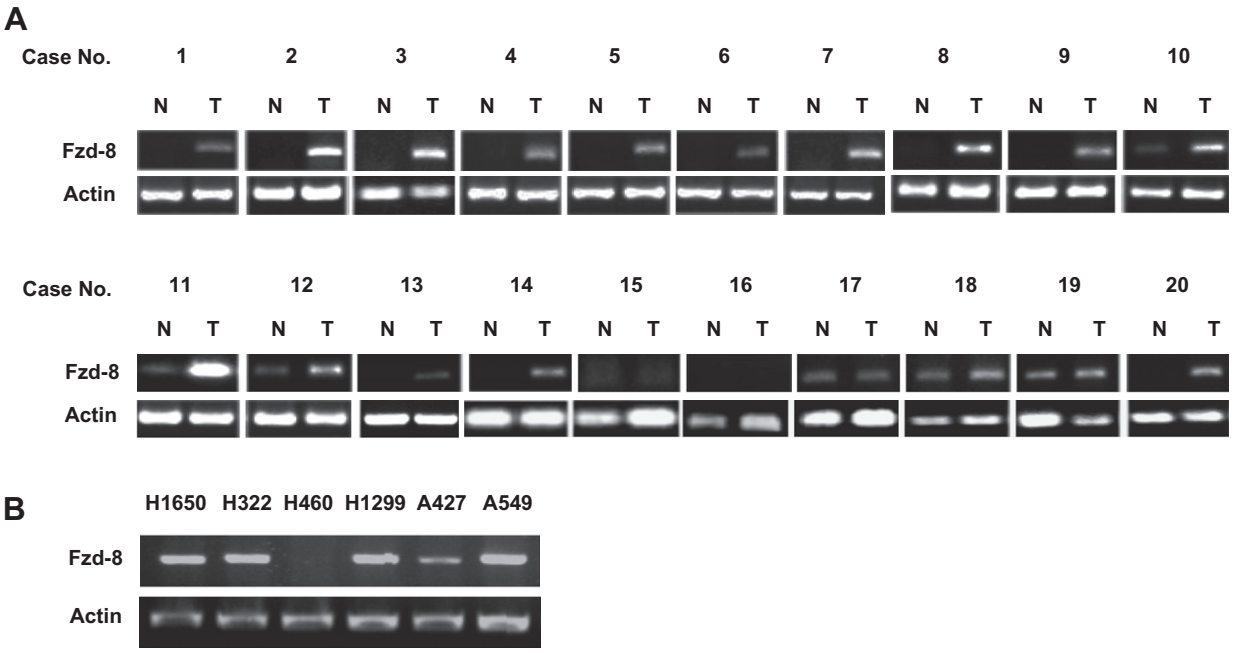

Fig. 1. (A) Semi-quantitative RT-PCR analysis of 20 freshly resected human lung cancer samples and their matched normal lung controls. The data shown represent tumor pairs (tumor (T) and normal (N)) of non-small cell lung cancer (NSCLC) samples. (B) Semi-quantitative RT-PCR analysis of human lung cancer cell lines. All PCR products were resolved on a 1.5% agarose gel. Actin was used as a loading control for all samples.

3.2. shRNA knock-down of Fzd-8 suppressed the canonical Wnt pathway in lung cancer cells

To investigate the function of Fzd-8 in human lung cancer, we examined effects of inhibiting Fzd-8 expression on the canonical Wnt signaling pathway in lung cancer cells. Two Fzd-8 targeted shRNAs with independent sequences were used to silence Fzd-8 mRNA expression in all experiments in order to avoid possible off-target effects produced by shRNA [15]. We first confirmed by using semi-quantitative RT-PCR that both stably transfected Fzd-8 shRNAs inhibited the Fzd-8 expression in those lung cancer cell lines (A549 and H1299) expressing the gene, whereas the non-silencing control shRNA had no effect (Fig. 2A). We then analyzed the cytosolic level of β-catenin protein, an important signature of the canonical Wnt activation [5,14], to explore effect of Fzd-8 shRNA on Wnt/β-catenin signal transduction. We found that cytosolic β-catenin protein levels were down-regulated after the treatment of both Fzd-8 shRNAs in those lung cancer cell lines that we examined (Fig. 2A), indicating that Fzd-8 functions as a positive regulator of the canonical Wnt/β-catenin signaling pathway in those cells. To further confirm the suppression of Wnt pathway by inhibition of Fzd-8 expression, we examined transcription of some downstream target genes of Wnt/β-catenin pathway such as Cyclin D1 and Survivin [5,14], in those lung cancer cells stably transfected with the Fzd-8 shRNAs. We found that the mRNA expression of both Cyclin D1 and Survivin was down-regulated after Fzd-8 knock-down (Fig. 2A). Taken together, these results suggest that Fzd-8 may be functionally important for aberrant activation of the canonical Wnt signaling pathway in human lung cancer cells.

3.3. shRNA knock-down of Fzd-8 inhibited proliferation of lung cancer cells and sensitized lung cancer cells to taxotere treatment in vitro

We next studied effects of inhibiting Fzd-8 expression on the cell survival in human lung cancer cells. Two weeks after either Fzd-8 shRNAs or non-silencing control shRNA transfection and subsequent G418 selection, we established stable transfectants of A549 and H1299 cells. Both MTS proliferation assay (Fig. 2B) and colony formation assay (Fig. 2C) showed that knock-down of Fzd-8 expression in those stable lines led to significant proliferative suppression when compared to the controls (MTS: $p < 0.005$, colony formation: $p < 0.001$, for both Fzd-8 shRNAs).

In addition, using A549 cells stably transfected with Fzd-8 shRNAs, we intended to examine the chemosynergic effect between Fzd-8 knock-down and chemo drug taxotere (Fig. 3). After these stable A549 cells were treated with moderate dose of taxotere (0.01 nM) for 3 days, we observed that the suppressive potential of taxotere was significantly enhanced by knock-down of Fzd-8 ($p < 0.001$ for both Fzd-8 shRNAs), indicating a potential future therapeutic role of the Fzd-8 inhibition in combination with current cytotoxic agents in lung cancer.

3.4. shRNA knock-down of Fzd-8 suppressed growth of lung cancer in vivo

Finally, we established xenograft mouse models with A549 cells stably transfected with control or Fzd-8 shRNAs. The stable lines were implanted into female BALB/c nude mice. Then tumor formation was monitored and tumor mass was measured every 3 days. We observed a significant reduction in tumor size and mass of the Fzd-8 shRNA tumors ($N = 5$ for both Fzd-8 shRNA-1 and shRNA-2 groups) compared to those of the control shRNA tumors ($N = 5$) (Fig. 4A, $p = 0.005$). After 4 weeks of tumor growth, mice were sacrificed and the tumors were collected for weight measurement. We found that tumor weight of both Fzd-8 shRNA treated groups was significantly less than that of the control shRNA treated group (Fig. 4B, $p < 0.001$). This result suggests that Fzd-8 may be a therapeutic target for lung cancer.

4. Discussion

Aberrant activation of the canonical Wnt signaling pathway has been demonstrated in numerous cancers including lung cancer

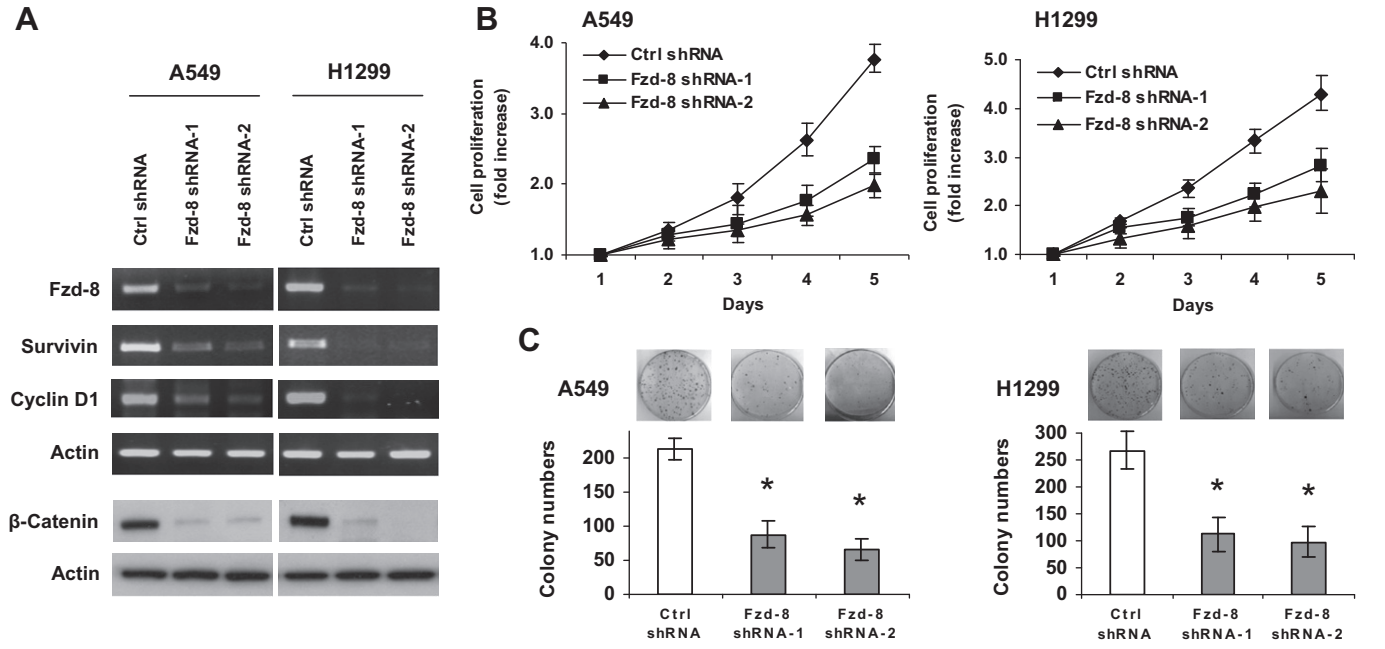

Fig. 2. Fzd-8 shRNAs suppressed lung cancer cell proliferation in vitro. (A) Two independent Fzd-8 shRNAs (shRNA-1 and shRNA-2) suppressed the canonical Wnt pathway in human lung cancer cell lines A549 and H1299. Semi-quantitative RT-PCR was used to examine mRNA expression and Western blot was used to examine cytosolic protein expression in these cell lines stably transfected with control or Fzd-8 shRNAs. Actin was used as a loading control for both semi-quantitative RT-PCR and Western blot. (B) MTS assay and (C) colony formation assay for A549 and H1299 stably transfected with control or Fzd-8 shRNAs. Experiments were performed in triplicate. Results are means ± S.D. (error bars). The images of colony formation assay were taken under a light microscope (20×).

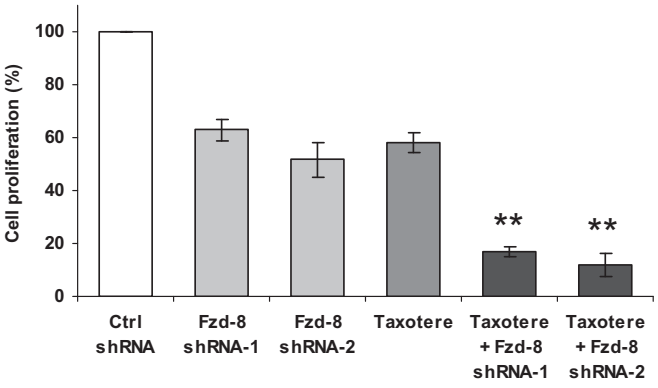

Fig. 3. Synergistic effect of Fzd-8 knock-down and taxotere in lung cancer cells. A549 cells stably transfected with control or Fzd-8 shRNAs were treated with moderate dose of taxotere (0.01 nM) for 3 days and cell survival was examined by MTS assay. These stable cells without taxotere treatment were included as controls. Experiments were performed in triplicate. Results are means ± S.D. (error bars).

[3,16]. Several upstream components of Wnt pathway were reported to be dysregulated in lung cancer. For example, both Wnt-1 and Wnt-2 were found to be up-regulated in NCSLC cell lines and primary tissues. Inhibition of Wnt-1 or Wnt-2 by siRNA or monoclonal antibodies induced apoptosis in NSCLC cell lines [4,5]. On the other hand, down-regulation of Wnt-7a was demonstrated in most NSCLC cell lines and primary tissues, suggesting that it might act as a novel tumor suppressor in lung cancer [6]. Co-expression of both Wnt-7a and Fzd-9 inhibited NSCLC cell growth, indicating a ligand-receptor role for these proteins [6]. Dvl, functioning downstream of the Fzd receptors as the mediator of Wnt signaling, has been reported being over-expressed in 75% of NSCLC tissues [7]. Inhibition of Dvl3 resulted in decreased TCF-dependent transcription and decreased cell growth [7]. Moreover, epigenetic silencing of the Wnt antagonists has been found to be important for aberrant activation of the canonical Wnt pathway in lung cancer [8–10]. Recent studies have demonstrated the involvement of several Fzd receptors such as Fzd-2, Fzd-4, Fzd-7 and Fzd-10 in different types of cancers [17–23]. However, it is still

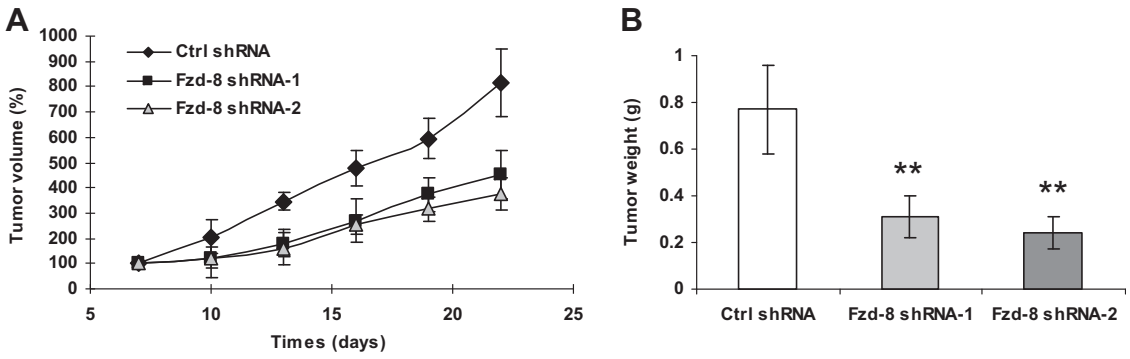

Fig. 4. Fzd-8 shRNAs suppressed lung cancer growth in vivo. (A) Tumor size was monitored and measured every 3 days after inoculation of A549 cells stably transfected with control or Fzd-8 shRNAs. Tumor volume was calculated by using the equation $x^2y/2$ (where $x < y$). (B) Tumor weight was measured at the completion of the experiment. Results are means ± S.D. (error bars).

unclear which Fzd receptors play important roles in the development of lung cancer.

In the present study, we examined the expression of Fzd-8 in human lung cancer. We also investigated whether Fzd-8 expression was associated with aberrant Wnt pathway activation and cell proliferation in lung cancer. We demonstrated a significant overexpression of Fzd-8 in primary lung cancer tissue samples when compared to their adjacent normal tissues, as well as in lung cancer cell lines that we examined. This observation suggests that Fzd-8 may be an important receptor for transducing Wnt signaling to activate downstream canonical Wnt cascade in human lung cancer. To test the possible functional significance of the Fzd-8 overexpression in lung cancer, we used shRNA and stable transfection technologies to knock down endogenous Fzd-8 expression in two lung cancer cell lines expressing the gene. We observed that knocking down Fzd-8 expression in these cells not only inhibited proliferation in vitro (demonstrated by both MTS and colony formation assays), but also suppressed tumor growth in vivo. Knocking down Fzd-8 expression was also accompanied by inhibition of the canonical Wnt signaling in these cells as evidenced by a decrease in the levels of cytosolic β-catenin and its downstream target genes such as Cyclin D1 and Survivin. These results indicate that Fzd-8 may play an important role in aberrant activation of the canonical Wnt pathway which is critical for the proliferation and survival of lung cancer cells. Taken together, our study suggests that Fzd-8 may be a good target for the development of therapeutics to treat lung cancer because of the nature of its membrane localization. A neutralizing monoclonal antibody strategy may be preferable in order to generate significant biological activity with minimal toxicity for the treatment of lung cancer. In addition, a recent report by DeAlmeida et al. [24] described an alternative strategy of developing potent inhibitor of Wnt signaling by using the extracellular cysteine-rich domain (CRD) of Fzd-8 fused to the human Fc domain. This soluble receptor fused protein was able to inhibit tumor growth in xenograft models, suggesting a potential therapeutic agent by targeting Fzd-8 mediated Wnt signaling. Such targeted agents may benefit lung cancer patients demonstrating a Fzd-8 tumor signature in the future. Moreover, we found that inhibition of Fzd-8 expression significantly sensitized lung cancer cells to the treatment of moderate dose of taxotere, a widely used chemo drug in the clinic to treat lung cancer. This result indicates a potential future treatment option of combining Fzd-8 inhibitors with current cytotoxic agents in lung cancer.

In summary, we propose that Fzd-8 is a putative promising therapeutic target for human lung cancer. Our findings laid the ground for the development of more effective new agents targeting Fzd-8 mediated signaling pathway for this deadly disease.

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