

Synergistic effect of targeting dishevelled-3 and the epidermal growth factor receptor-tyrosine kinase inhibitor on mesothelioma cells *in vitro*

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Abstract. It was previously revealed that Wnt signaling is activated in mesothelioma cells. Although epidermal growth factor receptor (EGFR) is expressed in mesothelioma cells, EGFR-tyrosine kinase inhibitors (TKIs) are not effective for mesothelioma treatment. However, in non-small cell lung cancer, the blocking of Wnt signaling has been identified to enhance the anticancer effect of EGFR-TKIs. To confirm the anticancer effect of blocking Wnt signaling in combination with EGFR-TKI treatment in mesothelioma, the present study evaluated the effect of simultaneous suppression of human dishevelled-3 (Dvl-3) expression with Dvl-3 small interfering RNA (siRNA) and of EGFR inhibition with gefitinib on mesothelioma cell viability. Mesothelioma cell lines with and without β -catenin gene expression were transfected with Dvl-3 siRNA and were cultured with gefitinib, and cell viability, colony formation and cell cycle analyses were performed. Dvl-3 siRNA downregulated the expression of Dvl-3 in mesothelioma cells. The combination of Dvl-3 siRNA with gefitinib acted synergistically to induce concomitant suppression of cell viability and colony formation, suggesting that inhibition

of Wnt signaling by downregulating Dvl-3 with siRNA and inhibiting EGFR with gefitinib leads to significant antitumor effects.

Introduction

Malignant mesothelioma is an asbestos-associated pleural malignancy that arises from serosal cells and presents a poor prognosis. Statistical surveys conducted by the Japanese Ministry of Health, Labor and Welfare reported that 1,410 fatalities were caused by mesothelioma in 2013 in Japan, double the number of fatalities in 1999 (www.mhlw.go.jp/toukei/saikin/hw/jinkou/tokusyuu/chuuhisyu15/dl/chuuhisyu.pdf). Although therapies including surgery, chemotherapy and radiotherapy have been adopted, the prognosis remains poor. Since the combination of an anti-folate reagent, pemetrexed, and cisplatin was demonstrated to prolong survival for longer than cisplatin alone (1), this combination has been frequently used, but its effects remain limited. Second-line therapy for tumor recurrence has not been established. Newer therapies based on an improved molecular understanding of mesothelioma are therefore required.

Wnt signaling, which activates a canonical pathway through β -catenin, is aberrantly activated in a wide range of tumors (2). Numerous types of tumor, including colon cancer, undergo aberrant activation of this canonical pathway and Wnt may also activate non-canonical pathways (2). Our previous study identified that Wnt signaling is activated in mesothelioma cells, and that blockade of Wnt signaling may be achieved with antibodies against Wnt-1 or -2, Wnt-1 or -2 small interfering RNAs (siRNAs), or dominant-negative dishevelled (Dvl), leading to suppressed viability or tumorigenesis of mesothelioma cells in athymic mice (3-7). Notably, the mesothelioma H28 cell line exhibits Wnt signal activity, which blocks apoptosis, without expression of β -catenin, due to a homozygous deletion of the β -catenin gene and activation of the aforementioned non-canonical pathways (8). Wnt signaling has been revealed to have a crucial function in maintaining cancer stem cells, which are highly resistant to chemotherapy (9).

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Abbreviations: Dvl, dishevelled; EGFR, epidermal growth factor receptor; GSK3 β , glycogen synthesis kinase-3 β ; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; TKI, tyrosine kinase inhibitor

Key words: mesothelioma, Wnt pathway, dishevelled-3, small interfering RNA, epidermal growth factor receptor, epidermal growth factor receptor-tyrosine kinase inhibitor

The epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib and afatinib are promising anticancer drugs for the treatment of patients with non-small cell lung cancer (NSCLC) with a specific EGFR mutation (10). EGFR has been revealed to be expressed in 68% of paraffin-embedded mesothelioma specimens (11). However, EGFR-TKIs alone are not effective in the treatment of mesothelioma (12). In NSCLC, tankyrase, an upregulator of the canonical Wnt signaling pathway, has been revealed to protect lung cancer cells from EGFR inhibition (13). Inhibition of β -catenin with EGFR-TKIs was reported to be able to enhance the anticancer effect or to overcome the resistance to EGFR-TKIs (14-16). In the present study, the effect of suppression of Wnt signaling with Dvl-3 siRNA and of inhibition of EGFR with gefitinib on mesothelioma cell viability were investigated.

Materials and methods

Cell lines and cell culture. Mesothelioma NCI-H28 (H28), NCI-H2452 (H2452) and MSTO-211H (211H) cell lines [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in RPMI-1640 complete medium (ATCC) containing 10% fetal bovine serum (FBS; ATCC) in a 75 cm² tissue culture flask in a 37°C 5% CO₂ incubator for 3 days. Medium was changed every 3 days. Once cells had reached 80% confluence, they were treated with 0.5% trypsin/0.2% EDTA solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and were passaged 1:4.

Western blot analysis. Cells were removed by scraping, washed with TBS [25 mM Tris-HCl (pH 8.0) and 150 mM NaCl] containing 0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 1,000 x g for 5 min at room temperature. Cells were lysed using mammalian protein extraction reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were centrifuged at 10,000 x g for 5 min at 4°C. Protein concentrations were determined using the PierceTM BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The supernatant was mixed with an equal volume of 4% SDS-containing 10% 2-mercaptoethanol. Whole cell lysate aliquots (20 μ g) were separated on 4-15% gradient SDS-polyacrylamide gels, subjected to 10% SDS-PAGE and were electrotransferred onto an ImmobilonTM polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for protein blotting. Blots were incubated overnight at 4°C with antibodies against Dvl-3 (1:1,000; 4D3; cat. no. sc-8027; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), β -catenin (1:1,000; cat. no. 610153; BD Biosciences, San Jose, CA, USA), EGFR (1:1,000; cat. no. sc-03; Santa Cruz Biotechnology, Inc.), glycogen synthesis kinase-3 β (GSK3 β) (1:5,000; cat. no. 9832; Cell Signaling Technology, Inc., Danvers, MA, USA) or phosphorylated (p)-GSK3 β (Ser⁹) (1:5,000; cat. no. 5558; Cell Signaling Technology, Inc.), and anti- β -actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) was used as an antibody for the reference protein. Antigen-antibody complexes were detected using an enhanced chemiluminescence blotting analysis system (GE Healthcare Life Sciences, Little Chalfont, UK).

Transfection with Dvl-3 siRNA. Dvl-3 siRNA (Stealth RNAiTM) was prepared by Invitrogen; Thermo Fisher Scientific, Inc. The

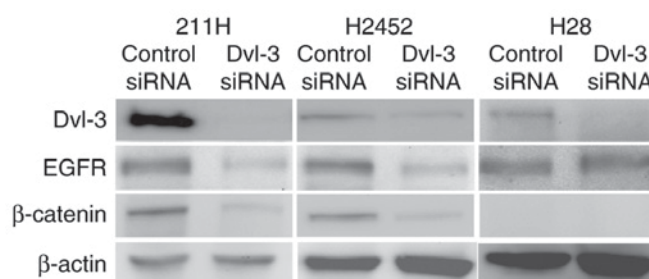


Figure 1. Downregulation of Dvl-3 expression in mesothelioma cells using Dvl-3 siRNA. Western blot analysis revealed that expression of Dvl-3 was downregulated in 211H, H2452 and H28 cells after 48 h of transfection with Dvl-3 siRNA, compared with cells transfected with control siRNA. 211H and H2452 cells exhibited expression of EGFR and β -catenin, whereas H28 exhibited no β -catenin expression due to homozygous deletion of the β -catenin gene. β -actin served as a loading control. Dvl-3, dishevelled-3; siRNA, small interfering RNA; EGFR, epidermal growth factor receptor.

sequence of the siRNA and the methods of transfection have been described previously (17). Briefly, cells were seeded onto 35-mm dishes at 1x10⁴ cells/dish, and were transfected 24 h later with 4 pmol siRNA using 4 ml LipofectamineTM 2000 (Thermo Fisher Scientific, Inc.). Cells were then incubated for 24 h at 37°C, washed once with PBS, then incubated with RPMI-1640 medium containing 10% FBS at 37°C for >24 h for western blot analysis and >14 days for colony formation assays on 35-mm dishes, or seeded onto 96-well plates for cytotoxicity assays.

Cytotoxicity assays. Cell viability was assessed using a modification of the MTT assay using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium and monosodium salt (WST-8) dye. Cells were seeded onto 96-well plates at 5x10³ cells/well 24 h after transfection with siRNA. Cell viability was estimated at 24, 48, 72 and 96 h after plating. WST-8 dye was added 2 h prior to the end of culture and absorbance was measured at 450 nm using a Multiskan JX instrument (Thermo Fisher Scientific, Inc.). Experiments were performed \geq 6 times. At 24 h after plating, cells were exposed to 5, 10 or 30 μ M gefitinib (Sigma-Aldrich; Merck KGaA) or dimethylsulfoxide (DMSO) as a control, and cell viability was estimated in the aforementioned manner. Gefitinib was dissolved in DMSO and controls for all experiments were created by adding equivalent volumes of DMSO. Each drug concentration was added to three replicate wells and each experiment was performed 4 times.

Colony formation assays. After 24 h of siRNA transfection, 400 cells were spread onto 35-mm dishes with RPMI-1640 medium and 10% FBS, with 5 μ M gefitinib or DMSO as a control. After 14 days, cells were stained with 0.5% methylene blue for 24 h at room temperature and colonies were counted visually. Colony assays were performed \geq 4 times and results are reported as the mean.

Cell cycle analysis. After 24 h of siRNA transfection, 5 μ M gefitinib or DMSO was added to the medium. A further 24 h later, cells were collected and a CycleTest PLUS DNA

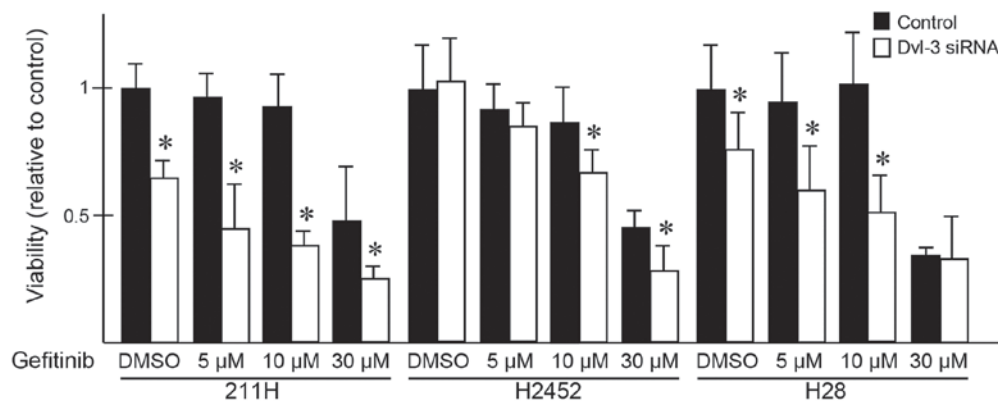


Figure 2. Synergistic suppression of mesothelioma cell viability following treatment with gefitinib and downregulation of Dvl-3. The suppressive effect on cell viability of Dvl-3 siRNA and an EGFR inhibitor, gefitinib, was examined. Cell viability was determined using a Cell Counting Kit-8. The results (ratio of viable cells transfected with Dvl-3 siRNA with or without gefitinib to those transfected with control siRNA and DMSO) at 48 h after the addition of gefitinib or DMSO are expressed as the mean \pm standard deviation. Cell viability of 211H, H2452 and H28 cells was synergistically suppressed when cells were transfected with Dvl-3 siRNA and treated with gefitinib. Experiments were performed ≥ 6 times. * $P < 0.05$ vs. control. Dvl-3, dishevelled-3; siRNA, small interfering RNA; EGFR, epidermal growth factor receptor; DMSO, dimethylsulfoxide.

Reagent kit (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's protocol. Cell cycle analysis was determined using a flow cytometer (BD FACSVerse™; version 1.0.3.2942; BD FACSuite software; BD Biosciences).

Statistical analysis. Results are expressed as the mean \pm standard deviation. Data between two groups were compared using a two-tailed unpaired Student's t-test. Analysis of variance (ANOVA), followed by Dunnett's test, was used to compare multiple groups. For cytotoxicity assays comparing concentrations of gefitinib, cells treated with DMSO were used as a control and the viability of other cells were compared using ANOVA followed by Dunnett's test. Viability of cells transfected with control siRNA was compared with that of those transfected with Dvl-3 siRNA using a two-sided Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using a commercial statistical software package (version 21; SPSS Statistics; IBM Corp., Armonk, NY, USA).

Results

Dvl-3 siRNA downregulates expression of Dvl-3 in mesothelioma cells. Mesothelioma 211H, H2452 and H28 cells express Dvl-3 and EGFR. Whereas H28 cells expressed no β -catenin due to a homozygous deletion of the β -catenin gene, 211H and H2452 cells did. At 48 h after transfection with Dvl-3 siRNA, expression of Dvl-3 was downregulated in 211H, H2452 and H28 cells (Fig. 1).

Downregulation of Dvl-3 and treatment with gefitinib suppresses the viability of mesothelioma cells synergistically. After 24 h of transfection with Dvl-3 siRNA or control siRNA, gefitinib or DMSO was added to the medium. Following transfection with Dvl-3 siRNA, the viability of H28 and 211H cells was significantly suppressed compared with that of the cells transfected with control siRNA, but the viability of H2452 cells was not (Fig. 2). At 48 h after the addition of gefitinib or DMSO, the viability of H28 cells transfected with control siRNA was not suppressed in the presence of 5 or

10 μ M gefitinib; however, the viability of H28 cells transfected with Dvl-3 siRNA was significantly suppressed following treatment with these concentrations of gefitinib compared with those treated with DMSO. The viability of 211H cells was significantly suppressed following treatment with 30 μ M gefitinib. At 5, 10 or 30 μ M gefitinib, the viability of 211H cells were significantly suppressed synergistically with Dvl-3 siRNA transfection compared with that of cells transfected with control siRNA. In H2452 cells, 10 and 30 μ M gefitinib significantly suppressed the viability of cells transfected with Dvl-3 siRNA, compared with that of those transfected with control siRNA.

Downregulation of Dvl-3 suppresses colony formation of mesothelioma cells. Colony counts of 211H, H2452 and H28 cells were significantly decreased following transfection with Dvl-3 siRNA, and were further significantly decreased following treatment with 5 μ M gefitinib, compared with respective controls (Fig. 3).

Downregulation of Dvl-3 and treatment with gefitinib induces G1 population increase. After 48 h of transfection with Dvl-3 siRNA, the population of 211H, H2452 and H28 cells in G1 phase tended to increase (Fig. 4). At a further 24 h after the addition of gefitinib to the medium, after 24 h of transfection with Dvl-3 siRNA, the population of 211H, H2452 and H28 cells in G1 phase tended to increase further, although these results were not statistically significant ($P > 0.05$; Fig. 4).

Downregulation of Dvl-3 decreases phosphorylation of GSK3 β . Phosphorylation of GSK3 β was decreased in 211H and H2452 cells following downregulation of Dvl-3 (Fig. 5). In H28 cells, expression of p-GSK3 β and total expression of GSK3 β were decreased.

Discussion

The results of the present study indicate that downregulation of Dvl-3 induced suppression of cell viability and that the addition of the EGFR-TKI gefitinib acted synergistically,

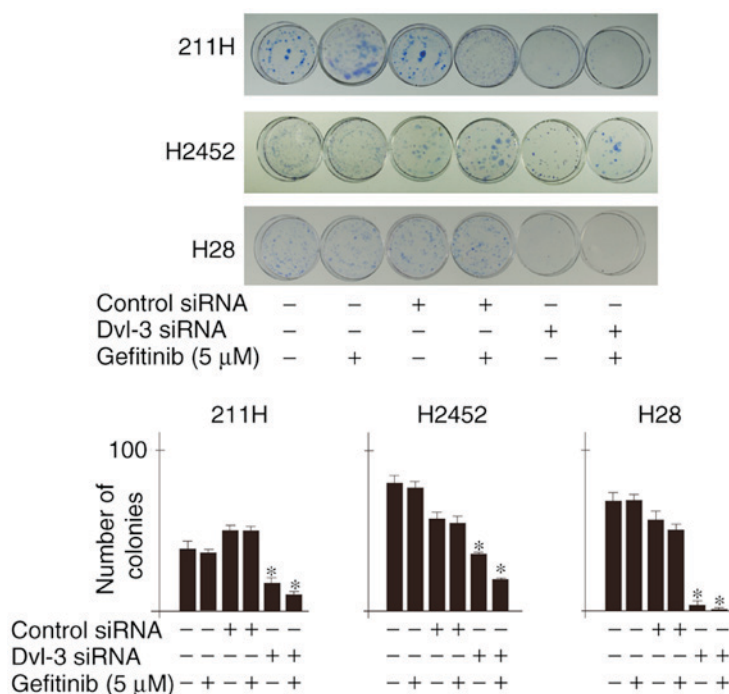


Figure 3. Inhibition of colony formation by downregulation of Dvl-3 and treatment with gefitinib. After 24 h of transfection with Dvl-3 siRNA, 400 cells were spread onto 35-mm dishes and colonies were counted 14 days later. Colony formation in 211H, H2452 and H28 cells was decreased following transfection with Dvl-3 siRNA, and was further suppressed by treatment with gefitinib, compared with cells transfected with control siRNA and those treated with gefitinib alone. The results of all experiments are expressed as the mean \pm standard deviation. Experiments were performed ≥ 6 times. * $P < 0.01$ vs. cells transfected with control siRNA. Dvl-3, dishevelled-3; siRNA, small interfering RNA.

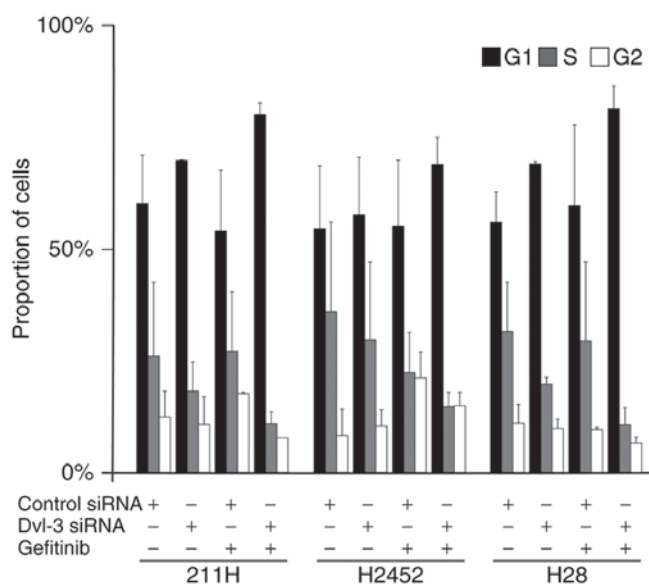


Figure 4. Aberration of cell cycle induced by downregulation of Dvl-3 with gefitinib. Cell cycle aberrations were induced by downregulation of Dvl-3 or transfection with Dvl-3 siRNA combined with treatment with 5 μ M gefitinib. The proportion of cells transfected with Dvl-3 siRNA and treated with gefitinib in the G1 phase tended to increase, compared with those transfected with control siRNA and those treated with gefitinib alone ($P > 0.05$). The results of all experiments are expressed as the mean \pm standard deviation. Dvl-3, dishevelled-3; siRNA, small interfering RNA.

resulting in colony formation with a tendency to persist in the G1 phase of the cell cycle. Of all pleural mesotheliomas, ~70% exhibit high levels of EGFR expression (11). Jänne *et al* (18) demonstrated that 10 μ M gefitinib suppressed the viability and

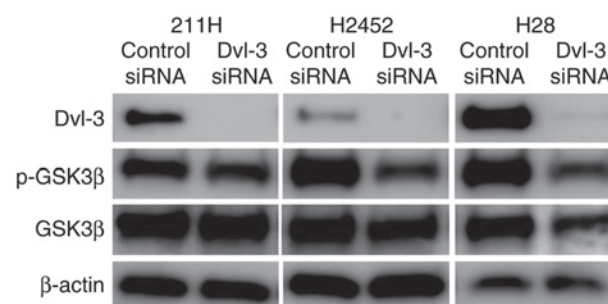


Figure 5. Decrease in phosphorylation of GSK3 β following downregulation of Dvl-3. Western blot analysis demonstrated that expression of GSK3 β and p-GSK3 β whole protein in 211H, H2452 and H28 cells after 48 h of transfection with Dvl-3 siRNA. β -actin served as loading control. GSK3 β , glycogen synthase kinase-3 β ; Dvl-3, dishevelled-3; p-, phosphorylated; siRNA, small interfering RNA.

colony formation of mesothelioma cell lines in soft agarose. It has been demonstrated that 10 μ M gefitinib exceeds the effective dose in NSCLC (13). In the present study, inhibition of Dvl-3 enhanced inhibition of viability at 10 μ M in all three mesothelioma cell lines. In H28 cells, downregulation of Dvl-3 suppressed cell viability, an effect which was enhanced 48 h after treatment with 5 or 10 μ M gefitinib. At 30 μ M gefitinib, H28 cell viability was markedly decreased, but it was not affected by downregulation of Dvl-3. Nutt *et al* (19) demonstrated that H28 cell viability was completely suppressed 72 h after the addition of 30 μ M gefitinib. A concentration of 30 μ M gefitinib is more toxic to H28 cells compared with a concentration of 5 or 10 μ M, and this toxicity may not be associated with signaling pathways affected by the downregulation of Dvl-3.

The aim of colony formation assay performed in the present study was to investigate the temporary effect of suppression of Dvl-3 combined with treatment with an EGFR-TKI on colony formation of mesothelioma cells. As colony formation was suppressed in the present study, suppression of Dvl-3 may be associated with the initial expansion of cells. A limitation of the present study is that the siRNA had no function after 14 days of transfection. It was confirmed that temporary transfection of siRNA did not suppress Dvl-3 expression after 14 days (data not shown). Future studies are required to examine colony formation using short hairpin RNA in order to elucidate the effect on other signaling pathways of continuous suppression of Dvl-3. In cell cycle analysis, 5 μ M gefitinib was used, and this dose did not inhibit cell viability effectively 24 h after the addition. Downregulation of Dvl-3 by siRNA usually induced G1 phase, which tended to be enhanced by gefitinib, although these results were not statistically significant. These results suggest that blockade of the EGF signaling pathway by gefitinib or other EGFR-TKIs, and of Wnt signaling by Dvl-3 suppression may be a useful combination for the treatment of mesothelioma.

p-GSK3 β (Ser⁹), which is the inactive form of GSK3 β and a regulator of Wnt signaling, and EGFR were revealed to be negatively associated with survival of patients with lung cancer, indicating that EGFR may phosphorylate GSK3 β into inactive p-GSK3 β (20). GSK3 β participates in various critical cellular processes, one of which is the formation of the β -catenin destruction complex (21). When Wnt signaling is not activated, GSK3 β is able to phosphorylate β -catenin, resulting in its ubiquitination. Dvl family members inhibit activation of GSK3 β and degradation of β -catenin, which is translocated to the nucleus and interacts with transcription factors, resulting in the expression of target genes (21). The results of the present study indicate that downregulation of Dvl-3 decreased phosphorylation of GSK3 β in 211H and H2452 cells. However, H28 cells without β -catenin expression exhibited a decrease in p-GSK3 β levels and total expression of GSK3 β following downregulation of Dvl-3. In 211H and H2452 cells, synergistic inhibition of cell viability by Dvl-3 downregulation and gefitinib may be associated with p-GSK3 β . However, the precise function of GSK3 β in EGFR and Wnt signaling pathways in mesothelioma cells requires further elucidation.

In NSCLC, Wnt signaling protects cells from EGFR-TKIs via tankyrase or β -catenin (13-16). An interaction between EGFR and Wnt signaling has been identified (22,23). Numerous studies reviewed in Paul *et al* (22) have demonstrated that downregulation of β -catenin leads to a decreased expression of EGFR, signal transducer and activator of transcription 3, cyclin D1, matrix metalloproteinase (MMP)2, MMP9 and protein kinase B. In mesothelioma cells, Wnt signaling and EGF signaling pathways may support each other against cytotoxicity.

Dvl proteins relay Wnt signals from receptors to downstream effectors, which activate either the canonical Wnt pathway or the β -catenin-independent non-canonical pathway, depending on the nuclear translocation of β -catenin (24). Previous studies have reported that the suppression of Dvl inhibits the viability or tumorigenesis of mesothelioma cells (3-7), and the cell viability of lung cancer (4). Furthermore, our previous studies demonstrated that mesothelioma cells express Dvl-3 and that the inhibition of Dvl-3 suppressed

mesothelioma cell viability, including that of H28 cells, which do not express β -catenin (8,17). This suggests that the activation of Wnt signaling in mesothelioma cells may utilize the β -catenin-independent non-canonical pathway. Zhao *et al* (25) demonstrated that Dvl-3 induced upregulation of p120-catenin, which is associated with cell viability, invasion and metastasis of lung cancer. In lung cancer cells, cytosolic transmembrane protein 88 was revealed to interact with Dvl family members independently of β -catenin, which promoted invasion and metastasis by activating p38-GSK3 β -Snail signaling (26).

The underlying molecular mechanism that led to these results may be attributed to a reciprocal interaction of the EGFR and Wnt signaling pathways. The function of Dvl-3 may differ among cell lines utilizing the canonical and non-canonical pathways. Notably, H28 cells do not express β -catenin, which is suspected to be associated with a different signaling pathway from those utilized by other mesothelioma cells. Although Dvl-3 is unable to affect EGFR directly in H28 cells, downregulation of Dvl-3 may inhibit other pathways to compensate for the negative effect induced by inhibition of the EGFR pathway in H28 cells. However, in order to fully understand these mechanisms, further studies are required. Furthermore, the mechanism of cross-talk between these pathways in mesothelioma cells remains to be elucidated.

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