Desmoplakin acts as a tumor suppressor by inhibition of the Wnt/β-catenin signaling pathway in human lung cancer

Linlin Yang1, Yuan Chen1, Tiantant Cui1, Thomas Knösel1, Qing Zhang2, Kai Frederik Albring2, Otmar Huber2 and Iver Petersen4,*

1Institute of Pathology, Jena University Hospital, Friedrich-Schiller-University Jena, Ziegelmühlenweg 1, 07743 Jena, Germany and 2Institute of Biochemistry II, Jena University Hospital, Friedrich-Schiller-University Jena, Nonnenplan 2–4, 07743 Jena, Germany

Desmosomes are intercellular junctions that confer strong cell–cell adhesion, thus conferring resistance against mechanical stress on epithelial tissues. A body of evidence indicates that decreased expression of desmosomal proteins is associated with poor prognosis in various cancers. As a key component of desmosomal plaque proteins, the functional role of desmoplakin (DSP) in cancer is not yet elucidated. Here, we reported the anti-tumorigenic activity of DSP in non-small cell lung cancer (NSCLC). We found by DSP DNA methylation that DSP expression was downregulated in 8 out of 11 lung cancer cell lines and in 34 out of 56 primary lung tumors. Ectopic expression of DSP in the NSCLC cell line H157 significantly inhibited cell proliferation, anchorage-independent growth, migration and invasion and also increased the sensitivity of NSCLC cells to apoptosis induced by an anticancer drug, gemcitabine. Furthermore, overexpression of DSP enhanced expression of plakoglobin (γ-catenin), resulting in decreased T-cell factor/lymphoid enhancer factor (TCF/LEF)-dependent transcriptional activity and reduced expression of the Wnt/β-catenin target genes Axin2 and matrix metalloproteinase MMP14. In accordance, DSP suppression by small interfering RNA resulted in downregulation of plakoglobin and upregulation of β-catenin and MMP14. Taken together, these data suggest that DSP is inactivated in lung cancer by an epigenetic mechanism, increases the sensitivity to anticancer drug-induced apoptosis and has tumor-suppressive function, possibly through inhibition of the Wnt/β-catenin signaling pathway in NSCLC cells. The epigenetic regulation of DSP and its ability to increase the sensitivity to anticancer drug-induced apoptosis has potential implications for clinical application.

Introduction

Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer death in men in 2008 globally. Among women, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008 (1). The most devastating aspect of lung cancer is metastasis. Despite the ever-increasing advances in modern treatment modalities, including surgery, chemotherapy and radiotherapy, the presence of metastasis remains the major determinant of poor outcome, with an overall 5-year survival rate of 15% for all stages combined (2).

Local invasion and subsequent distant metastasis is a complex multistep process (3). The initial step in the metastatic cascade is the loss of cell–cell adhesion at the primary site. It was recognized quite early that changes in intercellular adhesion accompany tumor dedifferentiation and progression (4). Desmosomes are intercellular junctions that provide strong adhesion among cells. They are ubiquitously expressed in epithelia and cardiac muscle and play a critical role in the maintenance of epithelial tissue integrity. Functional impairment of desmosomes results in various human diseases, including skin disease and cardiomyopathy (5,6). Recently, studies suggest that desmosomes participate in the regulation of cell motility, growth, differentiation and apoptosis (7–9).

Desmoplakin (DSP), as founding member of the plakin family, is an obligate component of desmosomal plaques (10). Two isoforms of DSP have been reported so far, DSP I (322 kDa) and DSP II (259 kDa), both encoded by the DSP gene on human chromosome 6p24.3. DSP proteins are widely expressed in numerous tissues (11,12). They interact with plakoglobin (γ-catenin), plakophilins and intermediate filaments, providing the intimate link between desmosomal cadherins and the cytoskeleton (13,14). Several studies have suggested that reduction of desmosomes was associated with invasive behavior in tumor cells (15,16). Decreased desmosomal protein expression was found in breast cancer (17), oropharyngeal squamous cell carcinoma (18), cervical carcinoma (19), colorectal cancer (20) and pancreatic cancer (21).

However, so far, little is known about the function of DSP in human cancers, including lung cancer. Therefore, this study aimed to investigate the epigenetic regulation, the functional role and the clinical relevance of DSP in human lung cancer.

Materials and methods

Cell lines and cell culture

Human bronchial epithelial cells (HBECs) were purchased from Clonetics (San Diego, CA) and cultured in bronchial epithelial cell growth medium (BEGM) (Clonetics). Human lung cancer cell lines, including small cell lung cancer (SCLC: COLO668, CPC-N and H82) and non-small cell lung cancer (NSCLC: H23, H2030, H2228, H157, H26 and H2170) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). Two NSCLC cell lines H1299 and H2347 were kindly provided by Dr Bastians (Göttingen, Germany). These cells were grown in RPMI1640 medium (Biochrom AG, Germany) supplemented with 10% (vol/vol) fetal bovine serum and maintained in a humidified atmosphere with 5% CO2 at 37°C.

Demethylation tests

Lung cancer cell lines COLO668, CPC-N, H82, H23, H2030, H157, H26 and H1299 were plated and cultured in 10-cm dishes. At 50% confluence, 10 μM 5-aza-2′-deoxycytidine (Sigma Chemical Co., St Louis, MO) was added to the medium on days 0, 2 and 4. Cells were then harvested for total RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) analyses.

Real-time RT-PCR analysis

Total RNA was extracted from cells using the Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed into complementary DNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed as described previously (20). Primer sequences are shown in Supplementary data 1, available at Carcinogenesis Online. The relative expression value of DSP to glyceraldehyde-3-phosphate dehydrogenase in each sample was calculated and compared. The experiments were performed in triplicates.

Western blot analyses

Proteins from whole cell lysate were isolated as described previously (22). Cytoplasmic and nuclear proteins were separated and isolated using nuclear extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions. Twenty micrograms of protein were used for expression analysis of DSP I/II, plakoglobin, β-catenin, caspase-3, β-actin, β-tubulin and laminA/C according to standard western blot protocols. The information about the antibodies is presented in Supplementary data 2, available at Carcinogenesis Online.
To analyze the effect of DSP on anchorage-independent cell growth, soft agar counts on days 2, 3, 4, 5, 6 and 7 with a cell counter (Countess, Invitrogen). To confirm the results, the experiments were performed in triplicate wells three times.

Wound-healing, cell migration and invasion assays

DSP transfectants and control cells were cultured in six-well plates until full confluence. Cell monolayer was carefully wounded using sterile 200 µl pipette tips across the diameter of the wells and washed twice with fresh medium. Then, time-lapse photography of the wounded edges was performed under an inverted-phase microscope for 48 h.

In the migration assay, 2 x 10^6 cells were resuspended in 300 µl of RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and placed in the upper transwell chamber (8 µm pore size, BD Biosciences). The upper chamber was placed in a 24-well culture dish containing 1 ml of medium. After incubation for 24 h at 37°C, non-migrated cells on the upper membrane were removed with a cotton swab. Migrated cells on the bottom surface were fixed with methanol (~20°C) and stained with 0.5% crystal violet. Four fields of each well were photographed and the cell numbers were counted. In the invasion assay, Matrigel-coated transwell chambers (BD Biosciences) were used. Percentage invasion was calculated as the number of invaded cells in comparison with the number of migrated cells. All the experiments were performed in triplicate three times.

Gemcitabine treatment and apoptosis assay

Apoptosis assay was carried out after cells were treated with 0.5 µM gemcitabine for 48 h. Briefly, the cells were incubated with APOPercentage Dye Label (Bioscolor Ltd, County Antrim, UK) for 30 min at 37°C. Purple-red-stained cells were identified as apoptotic cells and the number of apoptotic cells per 100 cells was counted. The experiments were performed three times in triplicate wells.

Reporter gene assays

H157 wild-type cells (1 x 10^5) were transfected with the indicated constructs using Turbofect (Thermo Scientific, Rockford, IL) according to the manufacturer’s recommendations. The following amounts of expression vectors were used for transfections: 0.5 µg of pGL3-OT/OF reporter constructs (kindly provided by Bert Vogelstein), 0.25 µg or 0.5 µg of pRC/CMV/DSP I and 0.5 µg of pCS2+[l]-catenin/S33A expression vectors. To normalize transfection efficiency, 0.05 µg of pHRLnull (Renilla luciferase) was cotransfected. The amount of DNA for each transfection was adjusted by addition of empty pCS2+ or pRC/CMV vector. Firefly and Renilla luciferase activities were measured with the PR-luc assay system in a GloMax™ 96 microplate luminometer (Promega GmbH, Mannheim, Germany) 24 h after transfection (24). Each value was obtained by double measurement and subsequent normalization of firefly luciferase activities with Renilla luciferase activities.

Statistical analyses

To study the relationship between DSP DNA methylation and DSP protein expression, 2 x 2 contingency tables were set up and chi-square or Fisher’s exact test was performed. The differences among DSP transfectants, mock transfectants and parental cells were analyzed by two-tailed Student’s t-test.

### Table 1. Study cohort

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total cases</th>
<th>DSP Negative</th>
<th>DSP Positive</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>45</td>
<td>26</td>
<td>19</td>
<td>0.363</td>
</tr>
<tr>
<td>Woman</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>34</td>
<td>23</td>
<td>11</td>
<td>0.187</td>
</tr>
<tr>
<td>&gt;60</td>
<td>62</td>
<td>22</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>48</td>
<td>28</td>
<td>20</td>
<td>0.372</td>
</tr>
<tr>
<td>T3-T4</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N = 0</td>
<td>32</td>
<td>20</td>
<td>12</td>
<td>0.655</td>
</tr>
<tr>
<td>N &gt; 0</td>
<td>23</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1-G2</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>0.349</td>
</tr>
<tr>
<td>G3-G4</td>
<td>24</td>
<td>13</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Methylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>27</td>
<td>21</td>
<td>6</td>
<td>0.012*</td>
</tr>
<tr>
<td>Unmethylated</td>
<td></td>
<td>29</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>

*Statistically significant (P < 0.05)
Desmoplakin inhibits Wnt/β-catenin signaling

Results

Downregulation of DSP I/II in lung cancer

To evaluate DSP protein expression in lung cancer, we performed western blot analyses in 11 lung cancer cell lines. HBECs were used as control. As shown in Figure 1A, compared with HBECs, DSP expression was slightly reduced in H2228, H2347 and H2170 and strongly decreased in other cell lines.

In primary lung tumors, DSP protein expression was assessed by immunohistochemistry (Figure 1B). Cytoplasmic staining of DSP was only found in 22 out of 56 (39.3%) primary lung tumor samples. However, expression levels of DSP did not differ significantly with respect to tumor grade, tumor stage and lymph node status.

Downregulation of DSP is associated with DNA methylation

To explore whether the reduced DSP expression is associated with DNA methylation, the eight lung cancer cell lines with strongly reduced DSP expression were treated with the methyltransferase inhibitor 5-aza-2′-deoxycytidine. After 5-aza-2′-deoxycytidine treatment, DSP mRNA expression was detectable in all these cell lines (Figure 2A).

To further analyze the methylation pattern of DSP, we performed BS in the promoter region and intron 1 of the DSP gene. We found that 10 or 6 out of 11 lung cancer cell lines exhibited DNA methylation in the promoter region or intron 1, respectively. The methylation pattern was heterogeneous. In the promoter region, DSP DNA was fully methylated in COLO668, CPC-N, H23, H2347, H226 and H1299, whereas DSP DNA was partly methylated in H82, H2030, H157 and H2170. In the cell line H2228 with endogenous DSP expression, no methylation of DSP DNA was found (Figure 2B). In intron 1, 8 out of 11 lung cancer cell lines, including COLO668, CPC-N, H82, H23, H2030, H157, H226 and H1299, were methylated. Among them, COLO668 and H1299 were completely methylated, but CPC-N, H82, H23, H2030, H157 and H226 were only partially methylated (Figure 2C). DSP DNA was not methylated in H2170, H2228 and H2347 cells, where DSP protein was only slightly downregulated. In contrast, in normal control HBECs, DSP DNA was partially methylated in the promoter region but unmethylated in intron 1. These results suggest that the methylation status of both promoter region and intron 1 is important for the regulation of DSP transcription.

To evaluate the methylation status of DSP in primary lung tumors, MSP was only carried out using primers designed for the intron 1 region, because the primer designed from the promoter region did not work well. MSP was first performed in lung cancer cell lines to test the specificity of the primers (Figure 2D). The reliability of the MSP results was verified by direct DNA sequencing (Supplementary data available at Carcinogenesis Online).

In primary lung tumors, DSP DNA methylation was detected in 27 out of 56 (48%) samples. Decreased DSP protein expression was significantly correlated with DSP DNA methylation (P = 0.012; Table 1). Examples of MSP in primary lung tumors are shown in Figure 2E.

DSP inhibits tumor cell proliferation, clonogenicity, migration and invasion

To investigate the functional role of DSP in lung cancer cells, we stably transfected H157 cells with an expression vector encoding wild-type DSP I. Two DSP-positive transfectants named D24 and D31 were selected for further investigations. Expression levels were analyzed by western blotting (Figure 3A) and immunofluorescence microscopy (Figure 3B). Mock transfectant clones named E8 and E9 and the parent cells were used as controls. Consistent with the expression pattern of DSP in primary lung tumors, we confirmed the cytoplasmic membrane-associated localization of ectopically expressed DSP by immunostaining, as reported previously (25).

We assessed the effect of DSP on cell proliferation by determining cumulative cell number. A reduced cumulative cell number in DSP transfectants was found compared with negative controls (Figure 3C). Anchorage-independent growth is another characteristic feature of cancer cells. We used soft agar colony formation assay to test the ability of various cells to confer anchorage-independent growth. As shown in Figure 3D, smaller numbers of colonies were observed in the transfectants D24 and D31 compared with the control clones (P < 0.001), suggesting that DSP is able to suppress tumor cell anchorage-independent growth.

To investigate the effect of DSP on cell motility, monolayer wound-healing assays were carried out. Over a 48 h time course, DSP transfectants spread along the wound edges more slowly than control cells (Figure 3E). To confirm the results, transwell migration assays...
were performed. As shown in Figure 3F, the number of cells that migrated into the lower chamber was significantly reduced in DSP transfectants compared with that in controls \( (P < 0.001) \), indicating that DSP inhibits tumor cell migration.

Furthermore, a matrigel assay was performed to examine the invasive potential of the cells. It turned out that the number of cells that migrated through the matrigel membrane into the lower chamber was significantly decreased in DSP transfectants compared with that in control cells and the percentage invasion of DSP transfectants was significantly lower than that of control cells \( (P < 0.001, \) Figure 3F), suggesting that DSP inhibits the invasive potential of cancer cells.

**DSP enhances the sensitivity of lung cancer cell to gemcitabine-induced apoptosis**

To elucidate the potential influence of DSP on apoptosis in lung cancer cells, we performed an APOPercentage assay to detect gemcitabine-induced apoptosis. As shown in Figure 4A, the number of apoptotic cells among DSP transfectants was significantly higher than that among control cells \( (P < 0.001) \). Consistent with this observation, the levels of the cleaved p17 fragment of caspase-3 were much higher in transfectants compared with the levels in control cells (Figure 4B).

**DSP inhibits Wnt signaling possibly through upregulation of plakoglobin**

To evaluate the effect of ectopic DSP expression on other desmosomal components, we analyzed the mRNA expression of desmoglein 1–3, desmocollin 1–3, plakophilin 1–3 and plakoglobin (\( \gamma \)-catenin) because plakoglobin antagonizes Wnt/\( \beta \)-catenin signaling and suppresses \( \beta \)-catenin transcriptional activity(26,27). We found that plakoglobin was upregulated, whereas \( \beta \)-catenin was downregulated in DSP transfectants compared with mock transfectants (Figure 5A). Additionally, western blot analyses clearly revealed elevated plakoglobin protein levels in both cytoplasm and nucleus of DSP transfectants, whereas nuclear \( \beta \)-catenin was alleviated in DSP transfectants compared with controls (Figure 5B). To further investigate location of plakoglobin and \( \beta \)-catenin, we performed immunofluorescence staining. The elevated plakoglobin in both cytoplasm and nucleus could be seen. In contrast, we found that the cytoplasmic and membrane staining of \( \beta \)-catenin was slightly upregulated (Figure 5C).

To test whether these changes affect \( \beta \)-catenin signaling activity in DSP-transfected cells TOPflash/FOPflash reporter gene...
assays were performed. H157 wild-type cells were transfected with or without β-catenin S33A and increasing amounts of DSP. A dose-dependent reduction of reporter gene activity was detectable in DSP-transfected cells (Figure 5D). We further analyzed the expression of two classical Wnt/β-catenin target genes, including Axin2, involved in regulation of Wnt/β-catenin signaling, and matrix metalloproteinase MMP14, related to cancer cell migration and metastasis. Consistent with the results of the reporter gene assays, the mRNA expression of Axin2 and MMP14 was downregulated in DSP transfectants compared with control cells (Figure 5E). For further investigation, DSP I/II was knocked down in the DSP-positive cell line H2228 by RNA interference. Successful knockdown of DSP was confirmed by western blot and real-time RT-PCR (Figure 5F and 5G). In contrast to DSP ectopic expression, DSP knockdown led to reduced expression of plakoglobin, enhanced expression of β-catenin and increased MMP14 expression, whereas Axin2 expression was not altered (Figure 5G).

**Desmoplakin inhibits Wnt/β-catenin signaling**

*Fig. 3.* Effects of ectopic DSP expression on the proliferation, clonogenicity, migration and invasion of carcinoma cells. (A) DSP overexpression in stably transfected cell line H157 was confirmed by western blot analysis. E8, E9: two independent mock-transfected clones; D24, D31: two independent DSP-positive clones. (B) Detection of DSP by immunofluorescence microscopy after staining of cells with an anti-DSP antibody (left panels). Nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; middle panels) and the overlay (right panels) is shown for mock- (top panels) and DSP-transfected cells (bottom panels). Bars: 20 μm. (C) Cell growth curves of parent cell, mock transfectants and DSP transfectants. (D) Top panel: Inhibition of colony formation by DSP in soft agar test. Bottom panel: Quantitative analysis of colony numbers. Values are the mean ± SE of three independent experiments. (E) Cell motility was determined by wound-healing assays. The ability of DSP transfectants to close the wounded area was significantly slower compared with control cells. (F) Quantification of transwell migration and matrigel invasion assays as described in the Materials and methods (upper diagram); the percentage invasion of DSP transfectants was also evaluated (lower diagram). The presented data are the means ± SE from three independent experiments. **P < 0.001 when analyzed with the unpaired t-test.
L. Yang et al.

-actin was used as loading control. Detection of activated caspase-3 by western blot to confirm the elevated activity. (B) Experiments. ** and methods. The data shown are the mean ± SE of three independent experiments. **P < 0.001 when analyzed with the unpaired t-test. (B) Detection of activated caspase-3 by western blot to confirm the elevated apoptotic level in DSP-transfected cells. β-actin was used as loading control.

These results suggested that reexpression of DSP represses Wnt/β-catenin signaling activity by increasing plakoglobin levels, and this may contribute to inhibition of tumor cell growth, migration and invasion. Downregulation of DSP as detected herein in many lung cancer cell lines is assumed to contribute to an upregulation of at least a set of β-catenin-regulated genes.

Discussion

In recent years, identification and characterization of specific tumor-associated genes and their products have accelerated the development of molecule-targeting drugs (28–30). Our present findings establish that DSP has tumor-suppressive function, which is exhibited through inhibition of the Wnt/β-catenin pathway in lung cancer.

Previous reports demonstrated that loss of DSP is not a rare molecular event in human cancer (16,18,31). Recent genetic loss-of-function studies implied that loss of DSP is an early step in carcinogenesis (16,32). Consistent with these results, our study showed decreased expression of DSP in a majority of lung cancer cell lines and primary lung tumors. However, we did not find an association between DSP protein expression and clinicopathological features, possibly due to a relatively small clinical sample size.

In the next step, we explored the epigenetic regulation of DSP expression. DNA methylation has been recognized to contribute to the regulation of gene expression in normal mammalian development and cancer pathogenesis (33). Alterations in DNA methylation are more common than genetic mutations or deletions and frequently result in gene silencing (34,35). A body of evidence supports the opinion that DNA methylation is responsible for the downregulation of desmosomal genes. For instance, desmocollin 3 was found to be downregulated in breast cancer and in colorectal cancer due to DNA methylation (17,20). Functional loss of plakoglobin (γ-catenin) was caused by DNA methylation in human prostate cancer and renal cell carcinoma (36,37). In our study, BS and MSP demonstrated that DSP was methylated in the promoter region and/or in intron 1 in cell lines and primary lung tumors, indicating that epigenetic regulation is responsible for DSP gene silencing. Based on our knowledge, this is the first report providing evidence for DSP DNA methylation in human cancer.

We selected the cell line H157 to study the function of DSP based on the following reasons: (i) H157 does not express DSP endogenously; (ii) H157 cells bear mesenchymal phenotype and are more malignant compared with other lung cancer cell lines; and (iii) the transfection efficiency was much higher in H157 than in other cell lines. In line with previous studies showing that other desmosomal plaque proteins, such as plakophilin 2 and plakoglobin, inhibited tumor growth and metastasis in cancer (9), our in vitro experiments clearly demonstrated that overexpression of DSP led to significant reduction of lung cancer cell proliferation and anchorage-independent growth. The reduced migration and invasion capacity can be partially attributed to impaired cell proliferative capacity; however, during analysis, we found that percentage invasion was much lower in the DSP transfectants compared with mock transfectants, indicating that DSP inhibits the invasiveness/migration of NSCLC cells. This is the first report on the tumor-suppressive activity of DSP in human cancer cells.

Gemcitabine, a pyrimidine nucleoside analog, is widely used as a chemotherapeutic agent and it functions through induction of apoptosis in various carcinomas including NSCLC (38–40). H157 is resistant to gemcitabine-induced apoptosis (41). However, this disadvantage was overcome by forced expression of DSP in H157, indicating that DSP might sensitize NSCLC cells to drug-induced apoptosis.

A critical and challenging question is the mechanism through which DSP exerts its tumor-suppressive effects in lung cancer. In keratinocytes, decreased expression of DSP could increase cell proliferation associated with elevated phospho-extracellular-signal-regulated kinases (ERK)1/2 and phospho-Akt levels (8). However, we did not observe any effects of DSP on activation of epidermal growth factor receptor (EGFR), extracellular-signal-regulated kinases ERK1/2 or Akt (data not shown). In contrast, we found that ectopic expression of DSP resulted in upregulation of plakoglobin (γ-catenin) in both cytoplasmic and nuclear fractions. Like β-catenin, plakoglobin is a component modulating the Wnt signaling pathway and may compete with β-catenin to interact with T-cell factor/lymphoid enhancer factor (42). TOP/FOPflash reporter gene assays revealed that the activity of Wnt/β-catenin signaling pathway was suppressed by overexpression of DSP, which was further proven by downregulation of Wnt/β target genes. In line with a previous study providing evidence that plakoglobin inhibited tumor growth by serving as a negative regulator of Wnt/β-catenin pathway in NSCLC cells (43), our study suggested that DSP-induced upregulation of plakoglobin inhibits Wnt/β-catenin signaling, leading to cell growth inhibition and reduced invasive potential. However, the folds of changes in the expression of Wnt/β-catenin target genes were different, indicating a non-linear relationship between DSP, plakoglobin and Wnt/β-catenin signaling activity. The complexity of the Wnt/β-catenin signaling network may also explain the observation that DSP knockdown did not increase Axin2 expression, whereas MMP14 was upregulated.

In conclusion, this is the first study reporting that DSP acts as a tumor suppressor in human lung cancer. Our data suggest that DSP upregulates plakoglobin, thereby inhibiting the Wnt/β-catenin pathway. The epigenetic regulation of DSP and its ability to increase the sensitivity to anticancer drug-induced apoptosis imply the clinical application of DSP in treatment of patients with lung cancer.

Funding

The Deutsche Krebshilfe (108003); Interdisciplinary Center for Clinical Research (IZKF, Jena, doctoral fellowship for Linlin Yang).

Acknowledgements

We are grateful to Manuela Pacyna-Gengelbach, Nicole Deutschmann and Prof. Dr Manfred Dietel for their support, including the collection of lung cancer specimens, at the Pathology Institute of the Charité. We thank Prof. Dr Werner W.Franke and Prof. Dr Holger Bastians for providing material facilitating the study.

Conflict of Interest: None declared.
Fig. 5. Effects of altered DSP expression on plakoglobin, β-catenin, Axin2 and MMP14 expression. (A) Real-time RT-PCR showed altered expression of plakoglobin and β-catenin in DSP transfectants D24 and D31 compared with parent cell and mock transfectants. (B) Western blot showed (i) elevated plakoglobin protein in both cytoplasm and nucleus and (ii) alleviated β-catenin proteins in nucleus by western blot. Lamin A/C was used as a control for nuclear protein. β-Tubulin was used as a control for cytoplasmic protein. (C) Immunofluorescence staining of plakoglobin and β-catenin. Middle panel: Nuclei stained with DAPI; right panel: Overlay of plakoglobin or β-catenin with nuclei. DSP (–): Mock transfectant cells; DSP (+): DSP-transfected cells. Magnification: ×40. Yellow arrow: cytoplasmic staining; white arrow: nuclear staining. (D) β-catenin transcriptional activity was measured using the TOPFlash/FOPFlash reporter constructs pGL3-OT/OF as described in Materials and methods. DSP(–): pRC/CMV plasmid; DSP(+): 0.25 µg pRC/CMV/DSP plasmid; DSP(++): 0.5 µg pRC/CMV/DSP plasmid. (E) Downregulation of Axin2 and MMP14 mRNA expression in DSP transfectant cells. (F) Suppression of DSP expression in H2228 cell line by DSP–siRNA duplex. Cells transfected with universal scrambled siRNA were used as control. (G) Downregulation of plakoglobin and upregulation of β-catenin and MMP14 mRNA expression were detected after DSP knockdown.

References


Received February 8, 2012; revised June 18, 2012; accepted July 7, 2012