Aberrant hypermethylation and reduced expression of disabled-2 promote the development of lung cancers

XUE-MEI XIE1-2, ZI-YIN ZHANG3, LIAN-HE YANG1, DA-LEI YANG4, NA TANG1, HUAN-YU ZHAO1, HONG-TAO XU1, QING-CHANG LI1 and EN-HUA WANG1

1Department of Pathology, The First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang 110001; 2Department of Pathology, Chengdu Military General Hospital, Chengdu 610083; 3Department of Neurosurgery, Meishan People’s Hospital, Meishan, Sichuan 620010; 4Assisted Reproduction Center, Shengjing Hospital of China Medical University, Shenyang 110004, P.R. China

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Abstract. Disabled-2 (Dab2) is considered a tumor suppressor and is downregulated in cancers. We examined the promoter methylation status and expression levels of Dab2, and investigated their roles in the development of lung cancers. Methylation-specific PCR was employed to analyze the methylation status of Dab2 in 100 lung cancer tissues. The cytoplasmic and nuclear expression of the Dab2 protein was determined using western blot analysis. Demethylation treatment using 5-Aza-2-deoxycytidine (5-Aza-dC) was performed in three lung cancer cell lines. Dab2 expression was upregulated by Dab2 transfection or interrupted by Dab2 siRNA in lung cancer cells. Proliferative and invasive ability tests were performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) and a Matrigel invasion assay, respectively. The methylation rate of Dab2 was significantly higher in lung cancer tissues compared to normal lung tissues. Dab2 methylation correlated with the reduced nuclear and cytoplasmic expression of Dab2, as well as the TNM stage and lymphatic metastasis of lung cancers. Treatment with 5-Aza-dC was able to eliminate the hypermethylation of Dab2, enhance Dab2 expression, and inhibit β-catenin expression, and the proliferative and invasive ability of lung cancer cells. Upregulation of Dab2 expression reduced β-catenin expression and proliferation and invasiveness of lung cancer cells. However, interruption of Dab2 expression induced the opposite results. Dab2 methylation is common in lung cancers, and is one of the most important factors responsible for the reduced expression of Dab2. Aberrant hypermethylation and reduced expression of Dab2 promote the development of lung cancers.

Introduction

The abnormal activation of Wnt/Wingless signaling pathway has been confirmed to be related to tumorigenesis in many tumor types (1,2). When the Wnt signal is weak, β-catenin is incorporated in a destruction complex that contains glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), axin, and casein kinase 1 (CK1), which results in phosphorylation of β-catenin; phosphorylated β-catenin is then degraded by a ubiquitin-mediated proteasomal pathway (3). However, in human cancers, the dissociation of β-catenin from this destruction complex results in the accumulation of β-catenin in the cytoplasm and nucleus, then activates the target genes of Wnt pathway, such as cyclin D1 and c-myc (1,2,4). Axin, a key member of the destruction complex, can be recruited to the plasma membrane by low-density lipoprotein receptor-related protein (LRP) 5/6 co-receptors, which is facilitated by dishevelled. This translocation will induce axin dephosphorylation by protein phosphatase 1 (PP1), resulting in its degradation (5-7).

Disabled-2 (Dab2) is a member of the Mammalia/ Drosophila disabled gene family (8), and contains 2 isoforms (p67 and p96) (9). It is a widely expressed endocytic adapter protein, and a regulator of some receptor-mediated signaling pathways (2,10-12). Dab2 can stabilize axin by preventing its phosphorylation and destruction complex results in the accumulation of β-catenin. When the Wnt signal is weak, β-catenin from this destruction complex results in the accumulation of β-catenin in the cytoplasm and nucleus, then activates the target genes of Wnt pathway, such as cyclin D1 and c-myc (1,2,4). Axin, a key member of the destruction complex, can be recruited to the plasma membrane by low-density lipoprotein receptor-related protein (LRP) 5/6 co-receptors, which is facilitated by dishevelled. This translocation will induce axin dephosphorylation by protein phosphatase 1 (PP1), resulting in its degradation (5-7).

In this study, we examined the relationship among Dab2 promoter methylation status, reduced expression of Dab2, and clinicopathological characteristics in lung cancers. In addition, we regulated the methylation status or expression level of Dab2 in lung cancer cells in order to investigate the mechanisms of Dab2 in the regulation of proliferation and invasiveness.

Correspondence to: Professor Hong-Tao Xu, Department of Pathology, The First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang 110001, P.R. China
E-mail: htxu@mail.cmu.edu.cn

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Materials and methods

Patients and specimens. A total of 100 paired fresh samples of primary lung cancer and corresponding normal lung tissues were selected randomly from patients diagnosed with lung cancer who underwent surgery at The First Affiliated Hospital of China Medical University between 2010 and 2012. The age of patients ranged from 37 to 82 years, and the mean age was 60 years (58 men and 42 women). The details of tumors were listed in Table I. Tumors were classified according to the system of the World Health Organization (2004), and the TNM classification scheme of the International Union Against Cancer. The study was conducted according to the regulations of the institutional review boards at China Medical University. Fresh tissue samples were stored at -70˚C immediately following resection.

Cell lines. A549, H157, H1299 and H460 cell lines were obtained from the ATCC (Manassas, VA, USA). LTEP-a-2 (hereafter referred to as LTE), SPC and LK2 cell lines were obtained from the Cell Bank of the Chinese Academy (Shanghai, China). The BE1 cell line was kindly provided by Professor J. Zheng (Medical College of Beijing University, China) (19-21). A549 and LK2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM); other cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ humidified atmosphere.

DNA extraction and methylation-specific PCR (MSP) analysis. Genomic DNA was isolated from tissue samples or cells with a tissue/cell DNA extraction reagent kit (Biotek, Beijing, China) according to the manufacturer’s protocol. Bisulfite conversion of DNA was performed with the EZ DNA Methylation kit (Zymo Research, Beijing, China) according to the manufacturer’s instructions. The primers of the nested PCR were as follows: forward, AAAGGTAGTTTTTTGTAAAGGG; reverse, TAAACTTAATAACTCCCCCTCA (product length: 367 bp). First, bisulfite-treated DNA was amplified for 30 cycles: 95˚C for 5 min, followed by cycling at 95˚C for 30 sec, 52˚C for 30 sec, and 72˚C for 45 sec, with a final extension step at 72˚C for 10 min. Next, the nested PCR products were diluted 100 times, and amplified for 45 cycles with MSP primers: 95˚C for 5 min, followed by cycling at 95˚C for 30 sec, 52˚C for 30 sec, and 72˚C for 45 sec, with a final extension step at 72˚C for 10 min. The primers of MSP were as follows: methylated forward, GGATTTGTGAAACGAGTTTC; methylated reverse, CACCAACTAAAAACGATCG (product length, 168 bp); un-methylated forward, GGATTTGTGAAATGAAGTTT; un-methylated reverse, CACCAACTAAAAACAATCA (product length, 168 bp). Finally, the MSP products were electrophoresized on 2% agarose gels containing ethidium bromide and analyzed using a Bio-Imaging system (UVP, Upland, CA, USA).

Protein extraction and western blot analysis. We randomly selected 50 paired lung cancer and corresponding normal lung tissues, in which methylation status had been examined previously, and extracted nuclear and cytoplasmic proteins separately using the Nuclear and Cytoplasmic Protein Extraction kit.
Demethylation assay. We performed a demethylation treatment in A549, LTE and H1299 cells, which showed complete methylation of Dab2 in the MSP examination. Cells were seeded in 6-well plates and allowed to confluence for 24 h, then treated with 5-Aza-2-deoxycytidine (5-Aza-dC) at a concentration of 5 µM for 72 h. The medium was changed every day. Cells cultured in the routine medium without 5-Aza-dC served as negative controls.

Dab2 gene transfection and siRNA knockdown assay. The Dab2 expression vector pRK5-Dab2 was kindly provided by Professor P.H. Howe (The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA) (5,12). The A549, LTE and H1299 cells were transfected with pRK5-Dab2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The cells transfected with empty vectors and un-transfected cells served as negative controls. Dab2 siRNA, control siRNA and siRNA reagent system were purchased from Santa Cruz Biotechnology Inc. The siRNA interference of Dab2 was performed according to the manufacturer’s instructions.

Cell proliferation analysis. The A549 cells transfected with Dab2, interrupted with Dab2 siRNA, or treated with 5-Aza-dC, along with control cells, were grown in 96-well plates separately at a density of 2.0×10⁴ cells/ml. Every 24 h, adherent cells were harvested and analyzed using The CellTiter 96 Aqueous One Solution cell proliferation assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTS)] (Promega, Madison, WI, USA). The absorbance, which is directly proportional to the number of living cells in culture, was measured at 490 nm using a microplate reader.

Cell invasion assay. Cell invasive ability was examined using a 24-well Transwell with 8-µm pore polycarbonate membrane inserts (Corning Inc., Corning, NY, USA). The A549 cells of each experimental group and corresponding control groups were seeded on the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, Saint Louis, MO, USA) at a density of 5×10⁵ cells/well in serum-free DMEM medium. The DMEM medium with 10% FBS was added to the lower chamber (600 µl/well). After 30-h incubation, the cells remaining on the upper membrane were removed with PBS and cotton wool, whereas cells that had invaded through the membrane were fixed with paraformaldehyde and stained with hematoxylin.

The cells were then viewed and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis. The paired sample t-tests was performed to analyze the cytoplasmic and nuclear expression level of Dab2 in lung cancers and the corresponding normal lung tissues. The independent t-tests was used to evaluate the expression level of Dab2 in lung cancers between promoter methylated and unmethylated of Dab2 gene. The Pearson’s χ² test, or likelihood ratio test, was used to determine relationships between Dab2 promoter methylation and clinicopathological characteristics of lung cancers or absent expression of p96-Dab2. The Spearman’s correlation test was used to examine the correlations between protein expression levels and Dab2 methylation. Experiments of lung cancer cells were independently repeated 3 times. P-values <0.05 were considered statistically significant.

Results

Hypermethylation of Dab2 is common in lung cancers and correlates with clinicopathological parameters. In 100 lung cancer tissues, 58 cases (58.0%) showed complete methylation, and 35 cases (35.0%) showed incomplete methylation. However, in corresponding normal lung tissues, no case showed complete methylation, and 35 cases (35.0%) showed incomplete methylation (Fig. 1A). So, the methylation rate of Dab2 in lung cancers (93.0%) was significantly higher than that in corresponding normal lung tissues. T, lung tumor tissues; N, normal lung tissues; M, methylated; U, unmethylated. (B) The methylation status of Dab2 in A549, LTE, H1299, H157, H460, LK2, SPC and BE1 lung cancer cells. (C) Treatment with 5-Aza-dC eliminated the methylation status of Dab2 in A549, LTE and H1299 cells.
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... (P=0.019), TNM stage (P=0.007), and histological type (P=0.022), but not correlated with gender (P=0.962) or age (P=0.248) of the patients (Table I).

Dab2 is localized both in the cytoplasm and nucleus, and the reduced expression of Dab2 correlates with the promoter methylation of Dab2 gene. After examination of the methylation status, we selected 50 paired cases, to detect the cytoplasm and nucleus expression of Dab2. The expression of p67-Dab2 was observed both in the cytoplasm and nucleus of lung cancer and normal lung tissues. However, p96-Dab2 was expressed only in the nuclei of normal lung tissues, but lost in lung cancer tissues (Fig. 2A). The cytoplasmic or nuclear expression of Dab2 in lung cancers was significantly lower than that in normal lung tissues (for cytoplasmic expression: 0.151±0.109 versus 0.696±0.337, t=-10.836, P<0.001; for nuclear expression: 0.337±0.181 versus 0.901±0.384, t=-10.726, P<0.001) (Fig. 2B).

Moreover, cytoplasmic expression levels of Dab2 in lung cancer cases with Dab2 promoter methylation (0.136±0.103) were significantly lower than that in lung cancer cases without Dab2 promoter methylation (0.261±0.095; t=-2.992, P=0.021). The nuclear expression of Dab2 also showed similar results (0.301±0.158 versus 0.603±0.101; t=-4.532, P<0.001) (Fig. 2C). The Spearman's correlation tests confirmed that Dab2 promoter methylation was negatively correlated with Dab2 expression levels in the cytoplasm (correlation coefficient, -0.258, P=0.009) and in the nucleus (correlation coefficient, -0.298, P=0.003) in lung cancer tissues. The loss of p96-Dab2 in corresponding normal lung tissues also correlated with the promoter methylation status of Dab2 ($\chi^2=12.063$, P=0.001). However, Dab2 expression levels did not correlate with clinicopathological parameters of patients (data not shown).

Treatment with 5-Aza-dC enhances the expression of Dab2, and inhibits the expression of β-catenin and the proliferative and invasive abilities of lung cancer cells. The promoter of Dab2 was methylated in all lung cancer cell lines used in this study. Complete methylation was observed in A549, L17E and H1299 cells, whereas incomplete methylation was observed in BE1, H460, SPC, H157 and LK2 cells (Fig. 1B). The completely methylated cells were then treated with 5-Aza-dC for 72 h separately. The promoter methylation of Dab2 was successfully eliminated (Fig. 1C), and expression levels of Dab2 were increased significantly (P<0.05), whereas β-catenin were significantly reduced in lung cancer cells (P<0.05) (Fig. 3A). Furthermore, the invasive cell number of 5-Aza-dC-treated A549 cells (9±2) was lower than that of untreated A549 cells (20±4) (P<0.05) (Fig. 3B and C). The growth rate of 5-Aza-dC-treated A549 cells was also reduced...
relative to that of untreated A549 cells at the second, third, and fourth days of detection (P<0.05) (Fig. 3D).

**Dab2 overexpression reduces the expression of β-catenin and inhibits the proliferative and invasive ability of lung cancer cells.** Both the nuclear and cytoplasmic expression of Dab2 was significantly enhanced after Dab2 gene transfection in A549, LTE and H1299 cells (P<0.05). Whereas, the expression of β-catenin was reduced (P<0.05) (Fig. 4A). The invasive cell number of A549 cells after Dab2 gene transfection (5±2) was reduced relative to the vector control A549 (23±4) and untransfected A549 cells (21±4) (P<0.05) (Fig. 5A and B). The growth rate of Dab2-transfected A549 cells was also reduced relative to that of vector control A549 and untransfected A549 cells at the second, third, and fourth days of detection (P<0.05) (Fig. 5C).

**Downregulation of the expression of Dab2 promotes the accumulation of β-catenin and enhances proliferation and invasiveness of lung cancer cells.** After interference with Dab2 siRNA, both the nuclear and cytoplasmic expression of Dab2 was weak or absent in A549, LTE and H1299 cells, respectively (P<0.05). The expression of β-catenin was increased (P<0.05) (Fig. 4B). The invasive cell number of A549 cells with Dab2 siRNA interference (60±5) was increased compared to the A549 cells treated with control siRNA (20±3) and untreated

Figure 3. The expression of Dab2 and β-catenin and the proliferative and invasive ability of lung cancer cells after treatment with 5-Aza-dC. (A) Compared with untreated control cells (5-Aza-dC-), the nuclear and cytoplasmic expression of Dab2 was increased, and the nuclear and cytoplasmic expression of β-catenin was reduced in the A549, LTE and H1299 cells after 5-Aza-dC treatment. Histone 3.1 and β-actin served as internal controls in the nucleus and cytoplasm, respectively. (B) Representative microscope fields of filters under the Matrigel are shown from 5-Aza-dC-treated A549 cells and untreated control cells (5-Aza-dC-), respectively (original magnification, x400). (C) The number of invasive cells in 5-Aza-dC treated A549 cells was reduced relative to that of untreated control cells (5-Aza-dC-) (bar, SD; *P<0.05). (D) The growth curves indicated that the growth rate of 5-Aza-dC-treated A549 cells was reduced relative to that of untreated A549 cells (5-Aza-dC-) (bar, SD; *P<0.05).

Figure 4. Nuclear and cytoplasmic expression of Dab2 and β-catenin in A549, H1299 and LTE cells with Dab2 transfection or Dab2 siRNA interference. (A) Both the nuclear and cytoplasmic expression of Dab2 in A549, LTE and H1299 cells with Dab2 gene transfection was significantly enhanced relative to the vector control cells or blank control cells. The expression, both nuclear and cytoplasmic, of β-catenin was markedly reduced. (B) The expression of Dab2 was absent or weak in A549, LTE and H1299 cells with Dab2 siRNA interference relative to the control siRNA or blank cells, whereas, both the nuclear and cytoplasmic expression of β-catenin was increased in A549, LTE and H1299 cells. Histone 3.1 and β-actin served as internal controls in the nucleus and in the cytoplasm, respectively.
A549 cells (19±4) (P<0.05) (Fig. 5D and E). The growth rate of A549 cells with Dab2 siRNA interference was also increased relative to A549 cells treated with control siRNA and untreated A549 cells at the second, third, and fourth days of detection (P<0.05) (Fig. 5F).

Discussion

Dab2 has been shown to be a widely expressed endocytic adaptor protein (10), and participates in a variety of physiological processes such as cell mitosis (22), endothelial cell differentiation (23), development of the central nervous system (24), and in the regulation of the TGF-β/Smad (12), and Wnt/β-catenin signaling pathways (13). Reduced expression of Dab2 will result in the activation of Wnt pathway. Furthermore, loss of Dab2 expression may facilitate the establishment of an autocrine TGFβ signalling loop, and promote TGFβ-stimulated epithelial-to-mesenchymal transition, and therefore increase the propensity for metastasis (25). Although downregulation of Dab2 has previously been demonstrated in other cancers (15,17,18,26), concrete explanations for this observation have yet to be well addressed.

We, for the first time, showed that Dab2 was expressed in the cytoplasm and nucleus using immunofluorescence (27), and western blot analysis, and its expression was significantly reduced in lung cancers, especially the p96 isoforms. We further showed that Dab2 overexpression inhibited the accumulation of β-catenin by Dab2 gene transfection, which conclusively inhibited proliferation and invasiveness of lung cancer cells. However, downregulation of the expression of Dab2 by Dab2 siRNA induced opposite results. We confirmed that reduced expression of Dab2 could induce the abnormal activation of Wnt pathway and promote the development of lung cancers.

Our study demonstrated that the methylation of Dab2 is common in lung cancers, similar to the reports in other tumors (23,28-30). Furthermore, the methylation of Dab2 was correlated with the differentiation, lymphatic metastasis, and TNM stage of lung cancers. Importantly, we found that the methylation of Dab2 was significantly correlated with reduced expression of the Dab2 protein in lung cancers. After treatment with 5-Aza-dC in A549, LTE and H1299 cells, which show complete methylation of the Dab2 promoter, we found the methylation of the Dab2 promoter was eliminated, and the expression of Dab2 was restored, which resulted in downregulation of β-catenin and the inhibition of the proliferative and invasive abilities of lung cancer cells. These results therefore demonstrated that the hypermethylation of Dab2 is a contributing factor in the reduced protein expression in lung cancers, and is also related to the development of lung cancers. So, the development of methods that could eliminate the methylation status of Dab2 or enhance the expression of Dab2 would offer potential therapeutic treatments for lung cancers.

In conclusion, the methylation of the gene Dab2 is common in lung cancers, and is one of the most important factors responsible for the reduced expression of Dab2. Furthermore, aberrant hypermethylation and reduced expression of Dab2 promote the development of lung cancers.

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