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# Suppression of non-small cell lung cancer proliferation and tumorigenicity by DENND2D

Bing Ling[a,1], Hongwei Zheng[a,1], Guobin Fu[a], Jingsong Yuan[a], Taiping Shi[b], Shiping Chen[c], Yu Liu[a], Yan Liu[a], Yan Cao[d], Shan Zheng[d], Suping Guo[a], Naijun Han[a], Yanning Gao[a], Shujun Cheng[a,**], Kaitai Zhang[a,*]

[a] State Key Laboratory of Molecular Oncology, Department of Etiology and Carcinogenesis, Cancer Institute (Hospital), Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, PR China
[b] Chinese National Human Genome Center, #3-707 North YongChang Road BDA, Beijing 100176, PR China
[c] Department of Cell Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, PR China
[d] Department of Pathology, Cancer Institute (Hospital), Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100021, PR China

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ABSTRACT

DENND2D was identified as being down-regulated in lung cancer using a lung cancer low-expression suppression subtractive hybridization (SSH) library. In this study, DENND2D down-regulation has been observed not only in non-small cell lung cancer (NSCLC) cell lines and lung squamous cell carcinoma (SCC) tissues, but also in immortalized human bronchial epithelial (IHBE) cell lines and precancerous lesions, indicating that the down-regulation of DENND2D may be an early event in lung cancer. The relative DNA copy number and mRNA and protein expression levels of DENND2D were determined in vitro, and they revealed a complicated regulatory network at the genomic, transcriptional and translational levels. Over-expression of DENND2D significantly suppressed the proliferation of NSCLC cells in vitro and in vivo by inducing apoptosis. These results indicate that DENND2D might function as a tumor suppressor-like gene to prevent the survival and expansion of cells with genetic damage through apoptosis mechanism, and absence of DENND2D might play a permissive role, as an early event, in tumorigenesis.


## 1. Introduction

DENN/MADD domain containing 2D (DENND2D; GenBank entry: AK026110) was identified using a lung cancer low-expression suppression subtractive hybridization (SSH) library that was previously used to identify differentially expressed genes [1]. The DENND2D gene maps to chromosome 1p13.3, spans chr1:111,729,803–111,747,031 and codes for a protein of 468 amino acids. DENND2D is part of the DENND2 family, which consists of 4 members. Unlike other members of the DENND2 family, DENND2D contains only the full DENN domain, with the upstream DENN (uDENN), core DENN and downstream DENN (dDENN) subregions. DENND2D is evolutionarily conserved across species, including primitive unicellular eukaryotes, fission yeast, filamentous fungi, and plants, but it is absent from budding yeast [2].

The Bloethner research group found that DENND2D mRNA was not present in 3 melanoma cell lines [3]. However, there are no reports regarding the role of DENND2D or DENN domain in any type of cancer. The DENN domain-containing protein MADD has been reported to inhibit apoptosis in leukemia and neuronal cells, but the death domain located at the C-terminal of the protein has been shown to suppress apoptosis [4–6]. DENND2D can serve as a guanine nucleotide exchange factor (GEF) stimulating the release of GDP from both Rab9A and Rab9B [2]. Rab GDPases are evolutionarily conserved across species, and they mainly function as regulators of membrane trafficking events [7,8]. Rab9A and Rab9B are members of the Rab family, which is mainly involved in the transportation of proteins between the late endosome and the trans-Golgi network [9–11]. Rab9 has not been reported to be associated with any type of cancer.

DENND2D is located on chromosome 1p. The aberrant deletion of chromosome 1p often occurs in NSCLC. This observation has been confirmed using cytogenetic analysis, allelotype

* Corresponding author at: Department of Etiology and Carcinogenesis, Cancer Institute (Hospital), Peking Union Medical College & Chinese Academy of Medical Sciences, P.O. Box 2258, Beijing 100021, PR China. Tel.: +86 10 6778 2323; fax: +86 10 8778 8726.
** Corresponding author at: Department of Etiology and Carcinogenesis, Cancer Institute (Hospital), Peking Union Medical College & Chinese Academy of Medical Sciences, P.O. Box 2258, Beijing 100021, PR China. Tel.: +86 10 6776 6703; fax: +86 10 6776 6703.
*E-mail addresses:* chengshj@263.net.cn (S. Cheng), zhangkt@cicams.ac.cn (K. Zhang).
[1] These authors contributed equally to this work.

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analysis and comparative genomic hybridization [12–17]. It remains unclear whether this chromosomal region contains genes with tumor suppression properties that are implicated in the biological and clinical behaviors of lung cancer. In this study, we demonstrate that DENND2D plays a functional role in non-small cell lung cancer.

## 2. Materials and methods

### 2.1. Patients and tissue samples

Total RNA was prepared from fresh lung SCC tissues and adjacent normal tissues obtained from 27 lung cancer patients. The purity of all tumor samples was greater than 50%. The patients included 26 males and 1 female, with a mean age of 59.7 years (44–76 years). For immunohistochemistry (IHC) analysis, 97 lung cancer patients were enrolled. These patients consisted of 72 males and 25 females. This group included 62 SCC patients and 35 adenocarcinoma (ADC) patients, with a mean age of 55.3 years (36–74 years). All of the patients underwent surgical treatment at the Cancer Institute & Hospital, the Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC). None of the patients received chemotherapy and/or radiotherapy before surgery. The experimental procedures were reviewed and approved by the ethics committees of the Cancer Institute & Hospital, PUMC and CAMS.

### 2.2. Antibody preparation and immunohistochemistry

The entire open reading frame of DENND2D was inserted into pGEX-4T-1 (GE Healthcare, Munich, Germany) and expressed as recombinant glutathione-S-transferase-DENND2D, which was purified and used to produce a polyclonal rabbit antibody against DENND2D by Professor Shiping Chen, Institute of Basic Medical Sciences, CAMS and PUMC, Beijing, China. Paraffin-embedded human tissues were deparaffinized in xylene and rehydrated in serially graded ethanol solutions. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution. Sections were boiled in EDTA (1 mM) antigen retrieval buffer (pH 9.0) for 40 min and then blocked using sheep serum. Sections were incubated with a mouse monoclonal antibody (1:1000) against DENND2D at 4 °C overnight. The secondary antibody (Zymed, San Francisco, CA, USA) was applied for 30 min at room temperature. This was followed by incubation with the PV-9000 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System kit (Zymed, San Francisco, CA, USA). Staining was visualized using 3,3′-diaminobenzidine tetrahydrochloride. The nuclei were counterstained using hematoxylin. IHC-stained sections were independently examined by two pathologists. Immunoreactivity scoring (IRS) criteria were used as previously described [18].

### 2.3. Gene cloning and plasmid construction

DENND2D full-length transcript (NM_024901) was amplified from a pool of human lung cDNAs (primers: 5′-CACTCCAGGGGCCATGGATG-3′ and 5′-GTCATTCTTATTCACCACAGCTC-3′) cloned in pcDNA3.1/mycHis(-)B (Invitrogen, Carlsbad, CA, USA) and sequenced.

### 2.4. Cell lines and cell culture

Three lung adenocarcinoma cell lines (A549, PAa and Glc82), three lung squamous cell cancer cell lines (H520, H2170 and LTEP) and three large-cell lung cancer cell lines (H460, H1299 and PG) were used. All nine cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS, Hyclone, Logan City, UT, USA). The IHBE cell lines MBE and YBE were established and maintained by our laboratory [19,20]. Primary cultured bronchial epithelial cell strains (PBEs) were cultured as previously described [21]. The first and second generations of the PBEs were harvested for total RNA extraction. All cells were cultured in a humidified 5% $CO_2$ atmosphere at 37 °C.

### 2.5. Transfection and infection

Plasmid transfection was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Generation of recombinant adenovirus and the infection was performed as previously described [22].

### 2.6. Cell proliferation assay

The rate of cell growth was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). The assays were performed at 24-h intervals (0–96 h) according to the manufacturer's protocol.

### 2.7. Foci-formation assay

Twenty-four hours after infection with Ad.DENND2D or the empty vector, 300 cells were seeded into 6-well plates. Cells were cultured for 10 days, fixed in cold methanol and stained with 0.5% crystal violet. Colonies larger than 0.5 mm in diameter were counted.

### 2.8. Tumorigenicity in nude mice

Female BALB/c athymic mice (nu/nu, 4 weeks old) were housed under aseptic conditions, and fed in accordance with the guidelines of the Animal Center of PUMC and CAMS. Mice were subcutaneously injected ($n = 6$; $5 \times 10^6$ cells per mouse). This experiment was repeated twice.

### 2.9. Immunoblotting

Lysates of A549, H520 and H1299 cells were prepared 72 h after adenovirus infection. Proteins (40 μg) were separated in 8% SDS–polyacrylamide gels (PAGE). Immunoblotting was performed as previously described [23]. Mouse monoclonal anti-DENND2D (1:2500) and rabbit polyclonal anti-cleaved PARP (1:1000) (Cell Signaling Technology, Beverly, MA, USA) were separately incubated with membranes at room temperature for 2 h. Blots were washed three times with phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 10 min, and they were then incubated with a 1:1000 dilution of goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) or GAPDH-HRP (1:10,000) (KangCheng, Shanghai, China).

### 2.10. Flow cytometry assay

H1299 cells were infected with Ad-DENND2D-Tet-On or negative controls. Cells were harvested 72 h after infection and stained and tested as previously described [23].

### 2.11. Statistical analysis

The statistical analysis of IHC data was performed using Fisher's exact test. Foci formation data were analyzed using the paired samples *t*-test. Other calculations were performed using one-way