Long non-coding RNA DANCR promotes HMGA2-mediated invasion in lung adenocarcinoma cells

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Abstract. Long non-coding RNAs (lncRNAs) have been reported to be key regulators in various types of cancer, including lung adenocarcinoma (LAD). The roles of the lncRNA differentiation antagonizing non-protein coding RNA (DANCR) and high mobility group AT-hook 2 (HMGA2) in LAD remain unclear. In the present study, it was revealed that the lncRNA DANCR was upregulated in LAD tissue and cell lines, compared with para-tumor tissue and a normal lung cell line. Additionally, elevated DANCR expression was associated with poor prognosis in the patients with LAD. Functionally, the study revealed that knockdown of DANCR inhibited invasion and HMGA2 expression in the LAD cell lines, SPCA1 and A549. Furthermore, HMGA2 was overexpressed in LAD tissue and in SPCA1 and A549 cells, compared with para-tumor tissue and a normal lung cell line. Inhibition of HMGA2 suppressed the invasive ability of SPCA1 and A549 cells, and DANCR promoted the invasive ability via regulation of HMGA2 in SPCA1 and A549 cells. The findings of the present study revealed that DANCR promoted the invasion of LAD cells by positively regulating HMGA2. Thus, a DANCR/HMGA2 axis may be a novel potential target in the molecular treatment of LAD.

Introduction

As the leading cause of cancer-related deaths worldwide, lung adenocarcinoma (LAD), which is the most prevalent subtype of lung cancer, accounts for ~14% of all neoplasms and is estimated to have produced >150,000 deaths in the last year (1). It is common for distant metastasis to occur in patients with LAD, including bone metastasis, cutaneous metastasis, thyroid metastasis and brain metastasis (2-5). Despite comprehensive treatment approaches, including chemotherapy, radiotherapy, surgical resection and molecular targeted therapy, the 5-year survival rate of LAD remains unsatisfactory (6,7). Therefore, understanding the molecular mechanisms and pathways of LAD metastasis is important to increase the treatment efficacy and improve the prognosis of patients with LAD.

Presently, whether DANCR regulates HMGA2 to mediate metastasis remains unclear.

Long non-coding RNAs (lncRNAs) are RNA transcripts with a length of >200 nucleotides. lncRNAs are involved in multiple cancer-related biological progresses, including proliferation, apoptosis, drug resistance, epithelial-mesenchymal transition and metastasis (8-12). The lncRNA differentiation antagonizing non-protein coding RNA (DANCR) is 1,189-bp nucleotides in length and is located at chromosome 4q12. DANCR has been reported to act as an oncogene in various types of cancer (13-16). Currently, few studies have investigated DANCR in LAD. Lu et al. reported that DANCR contributed to LAD progression by sponging microRNA (miR)-496 to modulate mTOR expression (17). It is well established that lncRNAs act via regulation of different downstream genes. The detailed mechanism of how DANCR functions in LAD requires further exploration.

High mobility group AT-hook 2 (HMGA2) is part of the HMGA protein family and is encoded by the HMGA2 gene located at chromosome 12q13-15. HMGA1a, HMGA1b and HMGA2 have analogous structures, and HMGA2 is well preserve evolutionarily (18,19). HMGA2 has been widely reported as a key regulator in multiple malignant tumor types, including gastric, thyroid and colorectal cancer, as well as esophageal squamous cell carcinoma and lung cancer (20-25). Meyer et al. reported that HMGA2 was overexpressed in non-small cell lung cancer (NSCLC) and served as a molecular marker for lung cancer (26). Xu et al. reported that angiogenin promoted the migration, invasion and proliferation capacity of squamous cell carcinoma of lung cells by directly upregulating HMGA2 (27). Li et al. demonstrated that lncRNA nuclear paraspeckle assembly transcript 1 facilitated cell growth and invasion by upregulation of HMGA2 in breast cancer (28). Presently, whether DANCR regulates HMGA2 to mediate metastasis remains unclear.
In the present study, DANCR and HMGA2 were overexpressed in LAD and were involved in the invasion of LAD cells. Additionally, HMGA2 was revealed to be a downstream gene of DANCR. DANCR promoted invasion via upregulation of HMGA2 in LAD cells.

Materials and methods

Patients and tissue samples. Specimens, 45 LAD tissue and paired para-tumor samples, were collected during tumorectomy at the Central Hospital Affiliated to Shenyang Medical College (Shenyang, China) between August 2012 and August 2017. Written informed consent was provided by all patients whose tissue was used in the present study. The Institute Research Medical Ethics Committee of Central Hospital Affiliated to Shenyang Medical College granted approval for this study. All 45 cases were diagnosed based on a definite pathological diagnosis and the clinical stages of these patients were determined according to the tumor node metastasis (TNM) classification (8th edition) of the International Union Against Cancer (UICC).

Cell culture. Human normal bronchial epithelial cell line 16HBE, and human LAD cell lines, SPCA1, A549, H1299 and H1975, were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). 16HBE cells were cultured in Airway Epithelial Cell Basal Medium (American Type Culture Collection, Manassas, VA, USA) A549 cells were cultured in F-12K medium (ATCC), and SPCA1, H1299 and H1975 cells were cultured in RPMI-1640 medium (ATCC). All culture media were supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 IU/ml penicillin (Baomanbio, Shanghai, China) and 100 mg/ml streptomycin (Baomanbio). All cell lines were cultured at 37˚C in a humidified atmosphere containing 5% CO2.

Reverse transcription and quantitative real-time PCR (qRT-PCR). The procedure was performed as previously described (11). Total RNAs from tissue specimens and cell lines were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The condition for reverse transcription was 37˚C for 15 min and 85˚C for 5 sec. PCR reactions containing SYBR detection system. The amplification condition was shown as below: 95˚C for 5 min, 38 cycles of 95˚C for 5 sec, 61˚C for 30 sec. GAPDH was used as an internal control to assess the expression levels of the DANCR and HMGA2. The following primer sequences were as an internal control to assess the expression levels of the HMGA2 in LAD cells.

Oligonucleotide transfection. Effective small interfering RNA (siRNA) oligonucleotides that targeted DANCR (accession no. NR_024031.2; siDANCR-01 and siDANCR-02) and HMGA2 (accession no. NM_001300918; siHMGA2-01 and siHMGA2-02) and a corresponding control siRNA (si-con) were synthesized by Guangzhou RiboBio Co., Ltd. Full length DANCR and HMGA2 fragments were amplified and cloned into the pcDNA3.1 vector to create DANCR and HMGA2 overexpression plasmids (oe-DANCR and oe-HMGA2) synthesized by Guangzhou RiboBio Co., Ltd. The sequences of the siRNAs were as follows: siDANCR-01, GGUAAAGUUAUUGACUA; siDANCR-02, GGUUUAUCAAUUGCUCUA; siHMGA2-01, GGGGACUUUAUUAUCU; siHMGA2-02, GGAAAGUGUCUUACA ACAA. When SPCA1 and A549 cells reached 70% confluence, the plasmids were transfected into the cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, as previously reported (29).

Transwell assay. The invasion assay was performed as previously described (30). Briefly, SPCA1 and A549 cells were seeded on the Matrigel-coated upper chambers of Transwell inserts (BD Biosciences, Franklin Lakes, NJ, USA). Culture medium with and without 10% FBS was supplemented into the lower and upper chambers, respectively, and incubated for 24 h. The subsequent day, the non-invaded cells were wiped from the membrane. Then the membranes were fixed in 90% alcohol and crystal violet staining followed. Five random fields were counted per chamber using an inverted microscope (Olympus Corp., Tokyo, Japan).

Western blot analysis. Total proteins were isolated using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA) and qualified using a bicinchoninic acid assay detecting kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Proteins samples (50 µg/well) were subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membranes. Anti-HMGA2 and anti-GAPDH antibodies (dilution 1:1,000 for anti-HMGA2, anti-GAPDH antibodies (dilution 1:1,000 for anti-HMGA2, cat. no. ab97276; and dilution 1:500 for anti-GAPDH; cat. no. ab205718; Abcam, Cambridge, UK) were applied and incubated with the membranes at 4˚C overnight. The following day, the membranes were incubated with secondary antibodies (dilution 1:2,000; cat. no. ab205718; Abcam) for 1 h at room temperature. Protein bands were detected on X-ray film using an enhanced chemiluminescence detection system.

Immunohistochemical (IHC) staining. The IHC procedure was performed as previously described (31). The LAD tissue specimens were processed as follows: 4% paraformaldehyde fixation, paraffin-embedding, sectioned to 4-µm thickness, deparaffinization, rehydration, hydrogen peroxide incubation, antigen retrieval, blocked in 10% goat serum (Bioworld Technology, Inc., St. Louis Park, MN, USA), primary antibody incubation (anti-HMGA2 and anti-GAPDH) at 4˚C overnight, secondary antibody incubation (goat anti-rabbit IgG H&L; Abcam) at 37˚C for 20 min, streptavidin-horseradish peroxidase complex incubation, dianobenzidine tetrahydrochloride.
Statistical analysis. All experiments were repeated in triplicate and all data from three independent experiments are presented as the mean ± standard deviation. GraphPad Prism software v5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) were used for statistical analysis. Association between DANCR and clinicopathological features of patients with LAD was analyzed using the Pearson’s Chi-square test. Survival analysis was performed using the log-rank test in GraphPad Prism v5.0. Differences between two groups were analyzed using the Student’s t-test or Student-Newman-Keuls method (S-N-K) method. P<0.05 was considered to indicate a statistically significant difference.

Results

DANCR is upregulated and associated with poor prognosis in patients with LAD. The expression of DANCR in the collected LAD tissue specimens was determined using qRT-PCR. DANCR was upregulated in the majority of LAD tissue specimens (39/45, 86.67%) compared with that in para-tumor tissue specimens (Fig. 1A and B). Additionally, DANCR was notably higher in patients with lymph node metastasis (N1 and N2) compared to patients without lymph node metastasis (N0; Fig. 1C). Furthermore, the association between the elevated DANCR expression and the clinicopathological features in patients with LAD was analyzed. As displayed in Fig. 1D and Table I, elevated DANCR was associated with a shorter survival rate (determined by Kaplan-Meier analysis), an advanced TNM stage (IIIa; P=0.013), lymph node metastasis (P=0.001) and a larger tumor size (P=0.023). Finally, the expression level of DANCR was assessed in a normal human bronchial epithelial cell line, 16HBE, and in four human LAD cell lines, SPCA1, A549, H1299 and H1975. DANCR was significantly upregulated in the four LAD cell lines (particularly in SPCA1 and A549) compared with 16HBE cells (Fig. 1E). Collectively, the results indicated that DANCR may act as an oncogene in LAD.

Downregulation of DANCR inhibits metastasis and HMGA2 expression in SPCA1 and A549 cells. The aforementioned results indicated that elevated DANCR was associated with lymph node metastasis. Loss-of-function experiments were then used to elucidate the potential role of DANCR in SPCA1 and A549 cell invasion. DANCR was knocked down using si-DANCR in SPCA1 and A549 cells (Fig. 2A). A Transwell assay was then performed to determine the changes in invasive ability. Downregulation of DANCR significantly inhibited the invasion of SPCA1 and A549 cells in the Transwell assay (Fig. 2B).

HMGA2 has been commonly reported as a metastatic gene in lung cancer. The expression changes of HMGA2 following DANCR knockdown were also evaluated in the
present study. As presented in Fig. 2C and D, downregulation of DANCR also inhibited HMGA2 expression at the mRNA and protein levels.

**HMGA2 is overexpressed in LAD and is involved in SPCA1 and A549 cell metastasis.** To further explore the function of HMGA2 in LAD, IHC was performed to detect HMGA2 expression in LAD tissue specimens. As presented in Fig. 3A, HMGA2 gradually increased with increasing pathological staging of LAD. Additionally, the expression of HMGA2 was determined in LAD cell lines. As revealed in Fig. 3B and C, compared with 16HBE, HMGA2 was overexpressed in LAD cell lines, SPCA1, A549, H1299 and H1975, as determined by western blotting and a qRT-PCR, respectively. Since DANCR and HMGA2 were revealed to be involved in SPCA1 and A549 cell metastasis, to further explore the function of HMGA2 in LAD, IHC was performed to detect HMGA2 expression in LAD tissue specimens. As presented in Fig. 3A, HMGA2 gradually increased with increasing pathological staging of LAD. Additionally, the expression of HMGA2 was determined in LAD cell lines. As revealed in Fig. 3B and C, compared with 16HBE, HMGA2 was overexpressed in LAD cell lines, SPCA1, A549, H1299 and H1975, as determined by western blotting and a qRT-PCR, respectively. Moreover, knockdown of HMGA2 using si-HMGA2 inhibited the invasion of SPCA1 and A549 cells in a Transwell assay (Fig. 3D and E).

**HMGA2 is a downstream effector in DANCR-facilitated metastasis in SPCA1 and A549 cells.** Since DANCR and HMGA2 were revealed to be involved in SPCA1 and A549 cell metastasis, we further explored their role in the formation of metastasis. As presented in Fig. 4A and B, downregulation of DANCR by si-DANCR-01 and si-DANCR-02 inhibited invasion of SPCA1 and A549 cells as determined by a Transwell assay. Moreover, knockdown of HMGA2 using si-HMGA2 also inhibited the invasion of SPCA1 and A549 cells as determined by a Transwell assay (Fig. 4C and D). These results indicated that HMGA2 is a downstream effector in DANCR-facilitated metastasis in SPCA1 and A549 cells.
invasion, the relationship between them was further explored. An increase and decrease of DANCR positively regulated HMGA2 expression at the mRNA and protein levels, which indicated that HMGA2 is a downstream effector of DANCR (Fig. 4A-C). Furthermore, siRNA and expression vectors were used to knockdown HMGA2 in DANCR-overexpressed A549 cells and overexpress HMGA2 in DANCR-silenced SPCA1 cells, as confirmed by qRT-PCR (Fig. 4D and E). Ultimately, a Transwell assay was used determine the role of HMGA2 in DANCR-mediated invasion. As revealed in Fig. 4F, upregulation of DANCR promoted the invasion ability of A549 cells, but the facilitative effect was attenuated by knockdown of HMGA2 (co-transfection of oe-DANCR and si-HMGA2). Conversely, knockdown of DANCR inhibited the invasion of SPCA1 cells, however, the suppressive effect was reversed by HMGA2 overexpression (co-transfection of si-DANCR and oe-HMGA2; Fig. 4G). The findings strongly indicated that DANCR promoted the invasion of LAD cells via positive regulation of HMGA2.

Discussion

A growing body of evidence has revealed that lncRNAs have important regulatory roles in various cellular behaviors and processes (32). DANCR, also termed ANCR, was initially identified as a non-coding RNA required to enforce the undifferentiated cell state within the epidermis (33-35). Currently, increasing evidence indicates that DANCR is involved in multiple biological processes, including stem cell differentiation, cell proliferation and cancer progression (13,16,29,36). Wang et al reported that DANCR promoted the proliferation, migration and invasion of NSCLC cell lines, SPC-A1 and H1299, via regulation of the tumor suppressor miR-758-3p (37). Zhen et al demonstrated that ectopic DANCR expression induced the proliferation and colony formation of lung cancer cells, whereas DANCR silencing promoted the opposing effect (38). In the present study, it was demonstrated that DANCR was overexpressed in LAD tissue specimens and in LAD cell lines compared with para-tumor tissue and a normal lung cell line, respectively. Additionally, elevated DANCR was associated with more progressive malignant phenotypes, such as advanced staging (P=0.013), larger tumor size (P=0.023), lymph node metastasis (P=0.001) and shorter survival time (P=0.024). This phenomenon indicated that DANCR may be a tumor initiator in LAD. Furthermore, the role of DANCR in the invasive abilities of SPCA1 and A549 cells was explored in loss-of-function experiments, which revealed that DANCR promoted the invasion of LAD cells.

HMGA2 protein is encoded by the HMGA2 gene that has at least five exons. HMGA2 participates in multiple nuclear processes including long-range chromatin interactions,
chromosome condensation, inhibition of nucleotide excision repair and regulation of gene transcription (39). HMGA2 is commonly reported as a transcriptional modulator that mediates motility and self-renewal in cancer stem cells (40). Through \textit{in vitro} and \textit{in vivo} study, Fan et al demonstrated that miR-543 inhibited the proliferation and metastasis of colorectal cancer cells by targeting KRAS, metastasis associated 1 and HMGA2 (41). Yang et al reported that HMGA2 mRNA and protein were highly expressed in metastatic breast cancer cells and that an inhibition of protease-activated receptor 1 suppressed HMGA2-driven invasion in breast cancer cells (42). In a lung cancer study, Gao et al demonstrated that HMGA2 is a target of miR-195 and that ectopic expression of HMGA2 increased the proliferation and migration ability of A549 cells (43). The expression and function of HMGA2 in LAD was also investigated in the present study. HMGA2 was highly expressed and promoted the invasion of the LAD cell lines, SPCA1 and A549. Knockdown of HMGA2 using siRNA inhibited the invasive abilities of SPCA1 and A549 cells. Additionally, the relationship between DANCR and HMGA2 was explored. DANCR regulated the expression of HMGA2 in a positive manner, and upregulation of DANCR promoted the invasion LAD cells, but the facilitative effect was attenuated by HMGA2 silencing. In opposing experiments, elevation of HMGA2 reversed the suppressive effect of DANCR silencing on LAD cell line invasion. This phenomenon indicated that HMGA2 is a key downstream effector in DANCR-mediated invasion of LAD cells.

It is well established that lncRNAs exert their functions via diverse mechanisms, including post-transcriptional regulation, genomic imprinting, chromatin remodeling and regulation of protein activity (44). The present study, only focused on the expression levels of DANCR and HMGA2, and the regulative effect that DANCR had on HMGA2. It was demonstrated that HMGA2 is a downstream effector involved in DANCR-induced invasion of LAD cells. However,
the detailed molecular mechanism and action sites between DANCR and HMGA2 still require further exploration. The present study illustrated that a DANCR/HMGA2 axis may be a novel target for treating LAD.

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Availability of data and materials
The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions
WJ conceived the experiments. NZ performed the experiments and analyzed the data. WJ wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
All LAD tissues and matched para-tumor tissues were in accordance with the ethical guidelines of the Central Hospital Affiliated to Shenyang Medical College and the Helsinki declaration. The ethics consents were signed by each patient before the study. All patients agreed that the data from their samples could be used for experimental studies and paper presentations.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


