Long noncoding RNA UPAT promoted cell proliferation via increasing UHRF1 expression in non-small cell lung cancer

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Abstract. Evidence indicates that long non-coding RNAs (lncRNAs) serve a critical role in the regulation of non-small cell lung cancer (NSCLC) progression. LncRNA Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) protein associated transcript (UPAT) has been identified to be overexpressed in a variety of types of cancer. The present study demonstrated that lncRNA UPAT expression was upregulated in NSCLC tissues and significantly associated with tumor size and Tumor-Node-Metastasis stage. Additionally, UPAT promoted NSCLC cell proliferation and G1-S phase transition in vitro. Furthermore, UPAT promoted NSCLC cell proliferation, partly via increasing UHRF1 expression and consequently epigenetically silencing RASSF1 and CDH13 transcription. In addition, the knockdown of UHRF1 partially decreased the promotion of cell growth and G1-S phase transition caused by UPAT overexpression. In conclusion, these data suggest that the lncRNA UPAT promotes the NSCLC cell proliferation and may be a potential therapeutic target of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most frequent and lethal malignancies worldwide; the 5-year survival rate is only 20% (1). Previous studies have indicated an increasing trend of NSCLC mortality in China in the previous 20 years, particularly in urban areas and among older (≥60 years old) people (2,3). An increasing number of studies have demonstrated the emerging roles of long non-coding RNAs (lncRNAs) in the tumorigenesis of NSCLC and have suggested their potential clinical applications as diagnostic markers and therapeutic targets in NSCLC (4,5).

LncRNAs are a class of non-coding RNAs that are >200 nucleotides in length (6). Dysregulation of lncRNAs contributes to the development and progression of several human diseases, notably lung cancer (7). For example, Hox Transcript Antisense RNA promotes proliferation, survival, invasion, metastasis and drug resistance in lung cancer cells (8). Metastasis-associated lung adenocarcinoma transcript 1 is an important target for anti-metastatic therapy in NSCLC (9,10). Antisense Noncoding RNA in the Inhibitors of cyclin dependent kinase 4 locus promotes NSCLC cell proliferation and inhibits apoptosis (11). Additional characterization of other lung cancer-associated lncRNAs will provide an improved understanding of their potential roles as therapeutic targets.

Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is overexpressed in various types of cancer, and was identified as a novel diagnostic marker of lung cancer (12). UHRF1, also known as ICBP90, was identified as a functional determinant of growth regulation genes, including p16INK4A and p14ARF, and the expression of UHRF1 is only detectable in proliferating cells, not in quiescent cells (13). Previous studies identified that UHRF1 serves a central function in epigenetic modulation during DNA duplication in the S phase (14-16). Taniue et al (17) demonstrated that lncRNA UHRF1 protein associated transcript (UPAT) stabilizes the UHRF1 protein by interfering with its ubiquitination and degradation, and promotes colon tumorigenesis (17).

The present study demonstrated that UPAT expression was also upregulated in NSCLC tissues. Additionally, UPAT significantly promoted cell growth and G1-S phase transition of NSCLC cells. Furthermore, lncRNA UPAT suppressed the expressions of Ras association domain-containing protein 1 (RASSF1) and Cadherin-13 (CDH13) by increasing UHRF1 expression, thereby promoting NSCLC cell proliferation. In conclusion, the data of the present study suggested that the lncRNA UPAT promoted the proliferation of NSCLC cells and may be a potential therapeutic target of NSCLC.

Materials and methods

Tissue collection and ethics statement. A total of 43 paired tumor tissues and matched normal tissues (>2.0 cm distance from the tumor edge) were collected from patients with
NSCLC (age range, 33-85 years old; mean age, 51.7 years old; 31 male and 12 female) who received surgical treatment between August 2011 and September 2015 at The Second Affiliated Hospital of Jiaxing University (Jiaxing, China). All experiments were approved by the Research Ethics Committee of Jiaxing University (Jiaxing, China). Written informed consent was obtained from all patients.

Cell culture. The human lung epithelial BEAS-2B cell line and NSCLC H1299, H1650, H358 and A549 cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 mg/ml streptomycin, and maintained at 37°C in humidified air containing 5% CO₂.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA of tissues and cells were extracted using of TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA by using the PrimeScript RT Master Mix Kit (Takara Biotechnology Co., Ltd., Tokyo, Japan). qPCR was carried out using SYBR Green remix (Takara Biotechnology Co., Ltd.) using an ABI Step One instrument (Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C, then 2 min at 72°C. The primers sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/ primerbank/; date of access, November 15, 2011). The sequences were as follows: UPAT forward, AACCAAGAGCCTGAAGACG, reverse, CTCACCTCCTTTCTCATTCT; UHRF1 forward, GCCACCCCAAAGTTCACATCTT and reverse, TGTGTC TATGACATTGCAGTC; RASSF1 forward, CCGCAGGT GATATTGAT and reverse, CACGAGGGCAGCATTTCTC T; CDH13 forward, AGTTGTTTCAATCAATCAGCCAG and reverse, CCTTACAGTCATGGAAGTCCAAG; GAPDH forward, TGTGGGCTAATGAGTGG and reverse, ACACCATGTTATCCGGGCTAAT. The relative amount of mRNA was calculated using the 2^ΔΔCq method (18). Gene expression was normalized by GAPDH. All data were obtained from three individual experiments.

Transfection of NSCLC cells. UPAT and UHRF1 siRNAs were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequence of UHRF1 was AAACAGAUG GAGGAGCCGCA, and the siRNA sequence of UPAT was AGGGAGTGAGAGGGAAATGT. A549 cells (1x10^5 cells/well) were seeded in a 6-well culture plate containing complete medium 24 h prior to transfection. The negative control scramble or UPAT siRNA (50 pmol/well) or UHRF1 siRNA (50 pmol/well) were transfected with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in H1299 cells according to the manufacturer's protocol. The full-length complementary DNA of UPAT was synthesized and subcloned into the pcDNA3 vector by Geneviz, Inc. (Suzhou, China), named pcDNA3-UPAT. The empty pcDNA3 vector (8 μg) or pcDNA3-UPAT (8 μg) were transfected with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in H1299 cells according to the manufacturer's protocol. At 24 h after transfection, the cells were treated and harvested.

Western blotting. A549 and H1299 cells (1x10^5) were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentrations were quantified using the BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities (20 μg) of protein were separated via SDS-PAGE (10%) and then transferred to poly(vinylide fluoride) membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 for 30 min at room temperature. This was followed by an incubation at 4°C overnight with primary antibodies: UHRF1 (sc-365392, 1:250 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); RASSF1 (sc-18722, 1:200 dilution; Santa Cruz Biotechnology, Inc.); CDH13 (sc-166785, 1:300 dilution; Santa Cruz Biotechnology, Inc.); and GAPDH (sc-47724, 1:500 dilution; Santa Cruz Biotechnology, Inc.). The membranes were washed and then incubated with goat anti-mouse horse-radish peroxidase-conjugated secondary antibody (sc-2005, 1:2000 dilution; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The membranes were then analyzed using ECL substrate (32106, Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. GAPDH was used as an endogenous protein for normalization. Protein bands were quantified using the Tanon-5200 Image Analyzer (Shanghai, China). Quantification of blots using Image J software (NIH, USA).

CCK-8 assay. The proliferation of A549 and H1299 cells were analyzed with a Cell Counting Kit-8 (CCK-8) (Dojin Molecular Technologies, Kumamoto, Japan). A549 and H1299 cells (1x10^5 cells/well) were seeded in 96-well plates at 37°C in humidified 5% CO₂ atmosphere. Following transfection treatment, 10 μl CCK-8 solution was added into each well and then incubated for another 4 h at 37°C. CCK-8 reagent was added at 0, 24, 48 and 72 h according to the manufacturer's protocol. The absorbance at 450 nm was measured using a microplate reader.

Cell cycle analysis. Following transfection treatment, A549 and H1299 cells were trypsinized and washed with cold PBS. The cells (1x10^5) were fixed in pre-chilled 70% ethanol at -20°C overnight. For measurement of DNA content, cells were stained with propidium iodide solution (50 μg/ml propidium iodide, 100 μg/ml RNase A, 0.05% Triton X-100 in PBS), and incubated at 37°C in the dark for 30 min. DNA content analysis by flow cytometry with a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of the cell population in each phase was calculated with the FlowJo FACs analysis software (version 7.0; Tree Star, Inc., Ashland, OR, USA).

Plasmid transfection and dual-luciferase reporter assay. The sequence of human UHRF1 (Gene ID: 29128) promoter (-2,000 bp) was obtained from the UCSC Genome Browser (uc002mbo.3, Human GRCh37/hg19; http://genome.ucsc.
The expression of the UPAT in 43 human NSCLC tissues

**Results**

UPAT is upregulated in human NSCLC tissues and cells. The expression of the UPAT in 43 human NSCLC tissues and matched adjacent normal tissues was first examined using RT-qPCR analysis. The results indicated that UPAT expression was significantly upregulated in NSCLC tissues compared with the control (Fig. 1A). The expression of UPAT was also analyzed in normal human lung epithelial BEAS-2B cell line and NSCLC H1299, H1650, H358 and A549 cell lines. GAPDH was used as an internal control. Data are presented as mean ± standard deviation. n=3. P<0.05 by analysis of variance. UPAT, Ubiquitin-like with PHD and RING finger domains 1 protein associated transcript; NSCLC, non-small cell lung cancer; qPCR, quantitative polymerase chain reaction.

Statistical analysis. Data are presented as mean ± standard deviation of three independent experiments. Differences between groups were analyzed by GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) with unpaired Student's t-test or one-way analysis of variance with a Tukey-Kramer post hoc test. The Pearson's Chi-square test was used to evaluate the difference between levels of UPAT expression in specimens and clinicopathological characteristics. P<0.05 was considered to indicate a statistically significant difference.

**Figure 1.** Relative expression of UPAT in NSCLC tissues and cells. (A) The relative expression of UPAT in NSCLC tissues (n=43) compared with corresponding non-tumor tissues (n=43). UPAT expression was examined by qPCR and normalized to GAPDH expression. The results are presented as the median fold change in tumor tissues compared with normal tissues, with interquartile ranges. *P<0.05 by Student's t-test. (B) UPAT expression was assessed by qPCR in the normal human lung epithelial BEAS-2B cell line and NSCLC H1299, H1650, H358 and A549 cell lines. GAPDH was used as an internal control. Data are presented as mean ± standard deviation. n=3. *P<0.05 by analysis of variance. UPAT, Ubiquitin-like with PHD and RING finger domains 1 protein associated transcript; NSCLC, non-small cell lung cancer; qPCR, quantitative polymerase chain reaction.

**UPAT promotes NSCLC cell proliferation and inhibits G1 arrest in vitro.** To explore the function of UPAT in NSCLC cells, the A549 and H1299 cell lines were selected for additional studies, as they exhibited the highest and lowest UPAT expression levels, respectively (Fig. 1B). Then, the overexpression plasmid and siRNA were used for transfection into the H1299 and A549 cell lines, respectively. The qPCR analysis indicated that the level of UPAT was increased in H1299 cells in tumor group (n=22). The median (2.13, in tumor group) was used as cut-off value. Then, the χ² analysis was performed to explore the potential associations between UPAT expression and clinical characteristics; the data revealed that the expression level of UPAT was significantly associated with tumor size, Tumor-Node-Metastasis stage and history of smoking, which suggests that the abnormal expression of UPAT contributed to the development of NSCLC (Table I).

To explore the function of UPAT in NSCLC cells, the A549 and H1299 cell lines were selected for additional studies, as they exhibited the highest and lowest UPAT expression levels, respectively (Fig. 1B). Then, the overexpression plasmid and siRNA were used for transfection into the H1299 and A549 cell lines, respectively. The qPCR analysis indicated that the level of UPAT was increased in H1299 cells in tumor group (n=22). The median (2.13, in tumor group) was used as cut-off value. Then, the χ² analysis was performed to explore the potential associations between UPAT expression and clinical characteristics; the data revealed that the expression level of UPAT was significantly associated with tumor size, Tumor-Node-Metastasis stage and history of smoking, which suggests that the abnormal expression of UPAT contributed to the development of NSCLC (Table I).

To determine the effect of UPAT on NSCLC cell proliferation, a CCK-8 assay was performed. The results indicated that the cell proliferation of H1299 cells transfected with pCDNA-UPAT was significantly increased compared with the control groups (Fig. 2C). By contrast, UPAT knockdown...
inhibited the A549 cell proliferation (Fig. 2D). These data indicated that UPAT markedly promoted the proliferation of NSCLC cells. The effects of UPAT on the cell cycle were additionally investigated. As indicated in Fig. 2E, overexpressing UPAT decreased the number of H1299 cells in the G1 phase and increased the number of cells in the S phase compared with the control. However, UPAT siRNA transfection caused a G1 phase cell-cycle arrest, with a significant increase in the percentage of A549 cells in G1 stage (Fig. 2F). These results suggest that the proliferation-inducing effects of UPAT are probably attributable to the promotion of the G1-S phase transition.

**UPAT inhibits RASSF1 and CDH13 expression.** A previous study demonstrated that UPAT is associated with UHRF1 and increases its protein level (17). Additionally, UHRF1 knockdown contributes to lower methylation levels of RASSF1 and CDH13 promoters in A549 NSCLC cells (19). To study the underlying molecular mechanisms and downstream targets involved in the effect of UPAT on cell cycle and growth, the mRNA levels of RASSF1 and CDH13, which are tumor suppressor genes involved in cell cycle and proliferation and are also inhibited by UHRF1 in NSCLC (20,21), were examined. The qPCR analysis demonstrated that RASSF1 and CDH13 mRNA levels were decreased in H1299 cells transfected with pCDNA-UPAT compared with the control (Fig. 3A). By contrast, UPAT knockdown increased the RASSF1 and CDH13 mRNA levels in A549 cells (Fig. 3B). Similarly, RASSF1 and CDH13 protein levels were decreased in H1299 cells transfected with pCDNA-UPAT but increased in A549 cells transfected with UPAT siRNA compared with the respective controls (Fig. 3C and D). Furthermore, it was also confirmed that UPAT overexpression increased the protein level of UHRF1 in the H1299 cells, but that UPAT knockdown decreased the protein level of UHRF1 in the A549 cells (Fig. 3C and D). These results suggested that UPAT may promote NSCLC cell proliferation partly through epigenetic silencing of RASSF1 and CDH13 transcription.

**UHRF1 overexpression is potentially involved in the oncogenic function of UPAT.** Luciferase vectors carrying the UHRF1 promoter (-2,000 bp) in front of the firefly luciferase gene coding region were constructed. As indicated in Fig. 4A, pCDNA-UPAT significantly increased the luciferase activity of the UHRF1 promoter; concurrently, treatment with UPAT siRNA suppressed the luciferase activity of UHRF1 promoter. To validate whether UPAT regulated NSCLC cell proliferation by increasing UHRF1 expression, rescue experiments were performed. A549 cells were co-transfected with pCDNA-UPAT and UHRF1 siRNA (Fig. 4B), and the CCK-8 and cell cycle assays results indicated that the knockdown of UHRF1 partially decreased the promotion of cell growth and G1-S phase transition resulting from UPAT overexpression.

### Table 1. Association between UPAT expression and clinicopathological characteristics of patients with non-small cell lung cancer (n=43).

| Characteristics          | All patients | UPAT low expression [< median] (%) | UPAT high expression [≥ median] (%) | P-value |
|--------------------------|-------------|-----------------------------------|------------------------------------|---------|
| Total                    | 43          | 21 (48.8)                         | 22 (51.2)                          | -       |
| Age, years               |             |                                    |                                    |         |
| <60                      | 26          | 13 (50.0)                         | 13 (50.0)                          | 0.85    |
| ≥60                      | 17          | 8 (47.1)                          | 9 (52.9)                           |         |
| Sex                      |             |                                    |                                    | 0.56    |
| Male                     | 31          | 16 (51.6)                         | 15 (48.4)                          | -       |
| Female                   | 12          | 5 (41.7)                          | 7 (58.3)                           | -       |
| Tumor size, cm           |             |                                    |                                    | 0.01    |
| <3                       | 18          | 13 (72.2)                         | 5 (27.8)                           | -       |
| ≥3                       | 25          | 8 (32.0)                          | 17 (68.0)                          |         |
| TNM stage                |             |                                    |                                    | 0.04    |
| I-II                     | 24          | 15 (62.5)                         | 9 (37.5)                           | -       |
| III-IV                   | 19          | 6 (31.6)                          | 13 (68.4)                          | -       |
| Metastasis               |             |                                    |                                    | 0.92    |
| No                       | 29          | 14 (48.3)                         | 15 (51.7)                          | -       |
| Yes                      | 14          | 7 (50.0)                          | 7 (50.0)                           | -       |
| History of smoking       |             |                                    |                                    | 0.03    |
| Never                    | 12          | 9 (75.0)                          | 3 (25.0)                           | -       |
| Ever                     | 31          | 12 (38.7)                         | 19 (61.3)                          | -       |

*The median expression level of UPAT was used as the cut-off. χ² test. *<p*<0.05. UPAT, Ubiquitin-like with PHD and RING finger domains 1 protein associated transcript; TNM, tumor node metastasis.
These results indicated that the effects of UPAT on NSCLC cell proliferation partially depended on its effect of increasing UHRF1 levels.

**Discussion**

UHRF1, which is overexpressed in several types of cancer, including breast, prostate and lung cancer, is a key regulator in the G1/S transition during the cell cycle (22). Previous studies have suggested that UHRF1/ICBP90 is associated with histone ubiquitination and DNA methylation in the context of tumor angiogenesis and silencing of tumor suppressor genes (23-26). UHRF1 binds hemi-methylated DNA through its SET- and RING-associated domains, triggering the recruitment of DNA (cytosine-5)-methyltransferase 1 and Histone deacetylase 1 (27,28). UHRF1 also mediates tumor
suppressing gene inactivation in NSCLC (19). UHRF1 knockdown contributes to lower methylation levels of RASSF1 and CDH13 promoters in A549 NSCLC cells (19). In the present study, it was identified that the expression of UPAT in NSCLC tissues was significantly increased compared with paratumor tissues, suggesting that UPAT serves a crucial role in NSCLC. Therefore, it was hypothesized that UPAT may exert its effects through increasing UHRF1 and epigenetically silencing RASSF1 and CDH13 transcription.

The data from the present study suggested that UPAT significantly promoted the proliferation of NSCLC cells by enhancing G1-S phase transition. Consistently, UPAT promotes colon tumorigenesis by inhibiting the degradation of UHRF1 (17). Additionally, in the present study, the downstream target genes of UHRF1 was analyzed by qPCR and western blotting assays, and the data indicated that RASSF1 and CDH13 were upregulated following UPAT knockdown. RASSF1 may form an endogenous complex with the activated Ras oncoprotein and integrate pro-growth pathways with pro-growth arrest/death pathways (21,29). CDH13 mediates cell growth arrest and regulates the cyclin-dependent kinase inhibitor 1 pathway (30). Loss of expression and aberrant methylation of the RASSF1 and CDH13 is observed in lung cancer (20,31). In the present study, it was demonstrated that RASSF1 and CDH13 served as tumor suppressors and were silenced by UPAT in NSCLC cells. Concurrently, UPAT also increased the expression of UHRF1.

To additionally confirm that UPAT exerted its effects through increasing UHRF1, rescue experiments were performed.
performed and it was identified that the knockdown of UHRF1 partially diminished UPAT-promoted cell growth and G1-S phase transition. These results revealed that the effects of UPAT on NSCLC cell proliferation were partially dependent on increasing UHRF1 expression.

In conclusion, the present study provides evidence that UPAT is upregulated in NSCLC tissues. The role of UPAT in the promotion of NSCLC cell proliferation may function partly via epigenetically inhibiting RASSF1 and CDH13 transcription by stabilizing UHRF1 expression and increasing the UHRF1-mediated methylation modification of these target genes. UPAT may serve as a potential target for the diagnosis and treatment of human NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FW conceived, organized and supervised the study. HW and DC performed the experiments. DC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Research Ethics Committee of Jiaxing University. Written informed consent was obtained from all patients.

Consent for publication

Written informed consent for publication was obtained from all patients for the publication of their data.
Competing interests

The authors declare that they have no competing interests.

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