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Original Article

Clinical implication of UCA1 in non-small cell lung cancer and its effect on caspase-3/7 activation and apoptosis induction in vitro

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Abstract: Since urothelial cancer associated 1 (UCA1) was discovered in human bladder cancer, it has been reported to be dysregulated expressed in various kinds of solid tumors. But the clinical role and the function of UCA1 in non-small cell lung cancer (NSCLC) remains incompletely understood. In this study, we mined the data of UCA1 expression in NSCLC from Oncomine, Gene expression profiling interactive analysis (GEPIA) and cBioPortal to analyze the contribution of UCA1 in the cancer initiation and progression of NSCLC. We also performed a series of in vitro experiments by using NSCLC cells to confirm the biological function of UCA1 in NSCLC, especially its effect on caspase-3/7 activity and apoptosis through RNA interference experiment. From Oncomine, the UCA1 levels were both up-regulated in lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC), as compared to non-cancerous controls. Higher levels of UCA1 pointed to a poorer overall survival in NSCLC, with the HR being 1.3. Only two genetic alterations, including amplification and deep deletion, were observed for UCA1 as provided by cBioPortal. Both MTS and Cell Titer-blue assays showed an accordant inhibitory effect of UCA1 siRNAs on the cell growth. In conclusion, lncRNA UCA1 might play a substantial role in the occurrence and development of NSCLC, especially in LUAD patients, which is partly due to its effect on caspase-3/7 activity suppression.

Keywords: UCA1, non-small cell lung cancer, caspase-3/7, apoptosis

Introduction

In the recent years, mounting evidence has showed that long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides, have become a novel star in the field of human cancers' occurrence and development [1-5]. Although lncRNAs are non-protein coding, they can modulate gene expression in other manners [6-9]. Urothelial cancer associated 1 (UCA1), the protagonist in this paper, is a member of lncRNAs. Since UCA1 was first discovered in human bladder cancer, it has been found to be dysregulated expressed in various kinds of solid tumors, such as esophageal squamous cell carcinoma (ESCC), ovarian cancer (OC), colorectal cancer (CRC), gastric cancer (GC), osteosarcoma, and so on [10-12]. And UCA1 was over-expressed in the most reported malignancies mentioned above [13-15]. The up-regulation of UCA1 was even analyzed to be associated with the clinicopathological characteristics of cancers, especially metastasis and survival [16-18]. Hence, UCA1 was thought to be an oncogene in certain kinds of tumors.

Each year, the incidence of lung cancer (LC) is increasing with about 1.8 million new cases, and the rate of cancer-related deaths from LC is over 25% in both males and females, which makes it one of the most common cancers in the world [19-21]. Non-small cell lung cancer (NSCLC) is the main subtype of LC [22-26]. Although recent studies have reported that high expression of UCA1 could promote the progression of NSCLC [27-29], the clinical function and the mechanism of UCA1 in NSCLC remains incompletely understood. Therefore, in this study, we mined the data of UCA1 expression in NSCLC from three public databases (Oncomine, Gene expression profiling interactive analysis (GEPIA) and cBioPortal) to comprehensive ana-

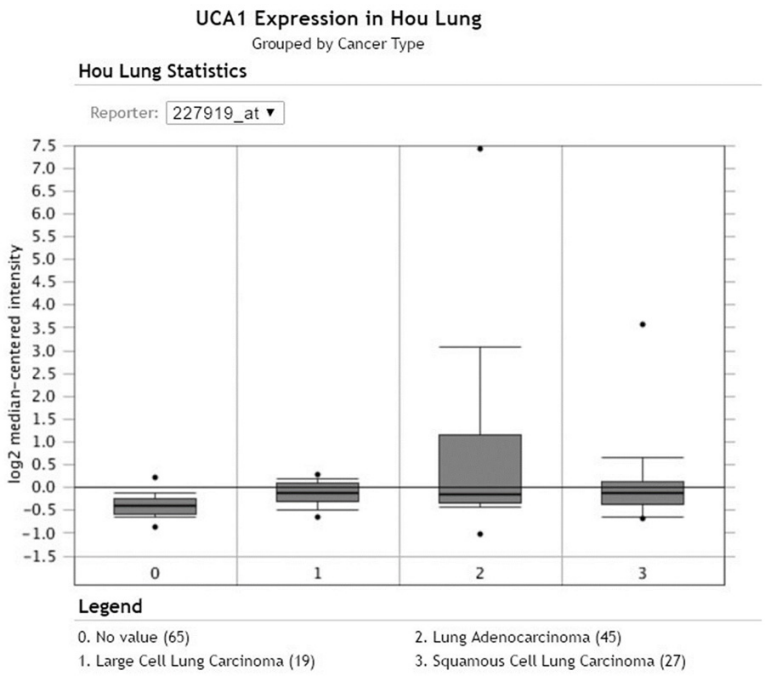

Figure 1. Expression level of UCA1 in the study of Hou Lung from Oncomine. The expression of UCA1 was provided by Oncomine. The study of Hou Lung consisted of 156 samples, and was performed with Human Genome U133 Plus 2.0 Array with 19,574 measured genes. 0: non-tumorous lung tissues (N=65); 1: large cell lung carcinoma (T=19); 2: LUAD (T=45); 3: LUSC (T=27).

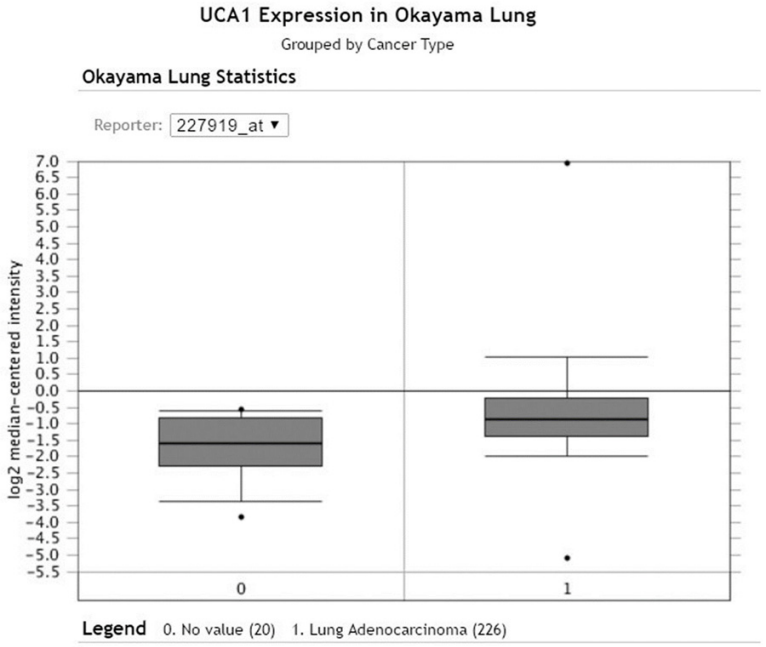

Figure 2. Expression level of UCA1 in the study of Okayama Lung from Oncomine. The expression of UCA1 was provided by Oncomine. The study of Okayama Lung consisted of 246 samples, and was performed with Human Genome U133 Plus 2.0 Array with 19,574 measured genes. 0: non-tumorous lung tissues (N=20); 1: LUAD (T=226).

lyze the contribution of UCA1 in the incidence and progression of NSCLC, which has not been attempted previously. Further, we performed a series experiments in vitro to confirm the biological function of UCA1 in NSCLC, especially its effect on caspase-3/7 activity and apoptosis by making RNA interference experiment.

Materials and methods

UCA1 expression in NSCLC tissues based on Oncomine and GEPIA public databases

The Oncomine™ Platform maintains gene expression signatures, clusters and gene-set modules for different diseases (https://www.oncomine.org/resource/login.html). On Oncomine v4.5, we first searched the UCA1 expression data in NSCLC. Then, the data with comparison of UCA1 expression between lung cancers and non-cancer controls were selected. The figures and calculated results were obtained from the website. Detailed information was recorded, including the sample size, subtypes of LC, detecting methods and mRNA numbers.

Gene expression profiling interactive analysis (GEPIA) could help analyze the RNA sequencing data of 9,736 tumors and 8,587 normal samples from The Cancer Genome Atlas (TCGA) and the GTEx projects, with a standard processing pipeline (http://gepia.cancer-pku.cn) [30]. (The expression data of UCA1, as well as its comparison in different stages and high/low survival, was obtained from GEPIA).

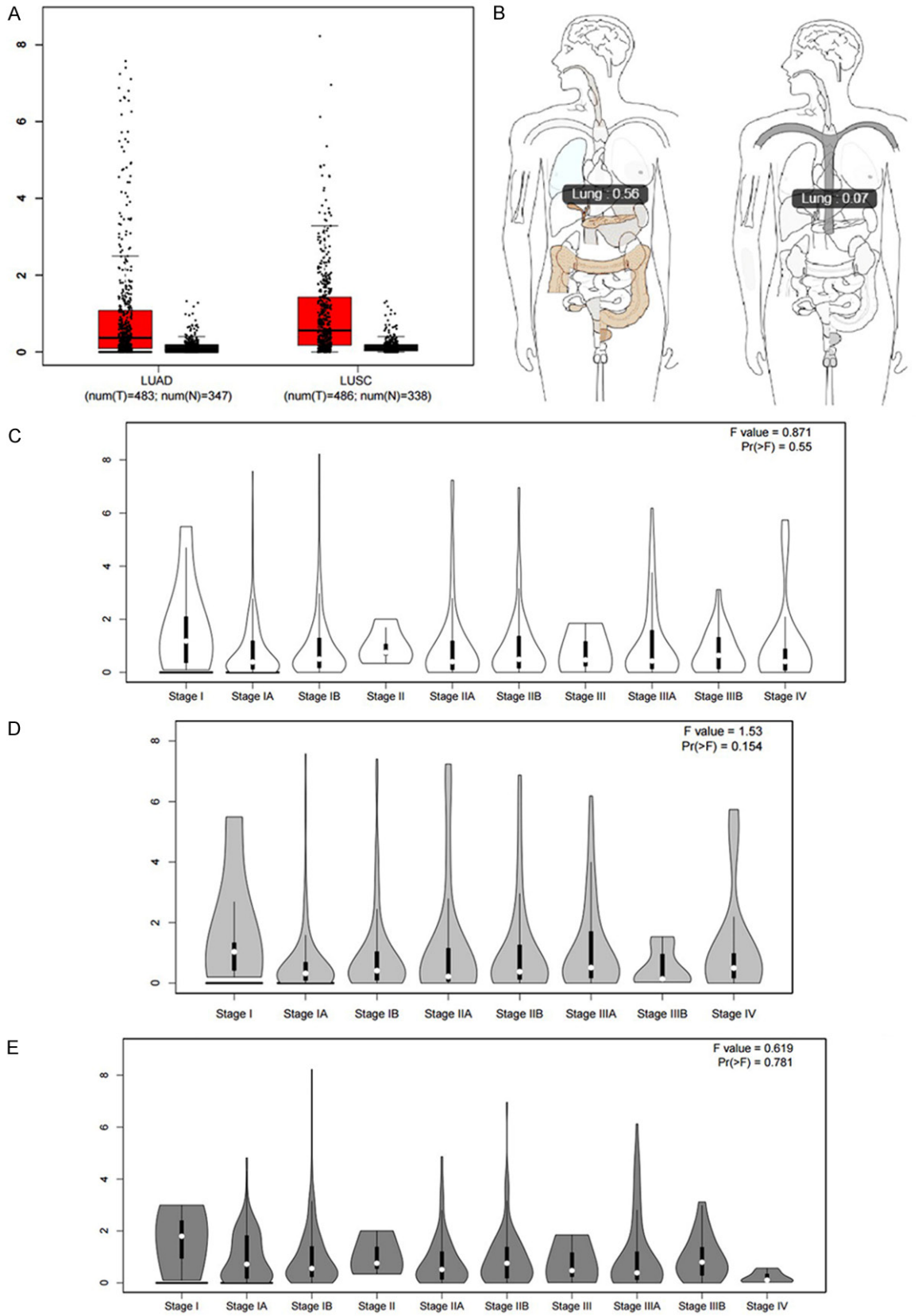

Figure 3. Expression level of UCA1 mRNA provided by GEPIA. The expression of UCA1 was provided by GEPIA. (A) Left: the expression of UCA1 in LUAD (T=483) and non-cancerous lung tissues (N=347); Right: the expression of UCA1 in LUSC (T=486) and non-cancerous lung tissues (N=338). Between LUAD and (B) UCA1 the median expression level showed in body maps. Left: non-small cell lung cancer (NSCLC); Right: non-cancerous tissues. The level of UCA1 in different clinical stages in NSCLC (C), LUAD (D) and LUSC (E).

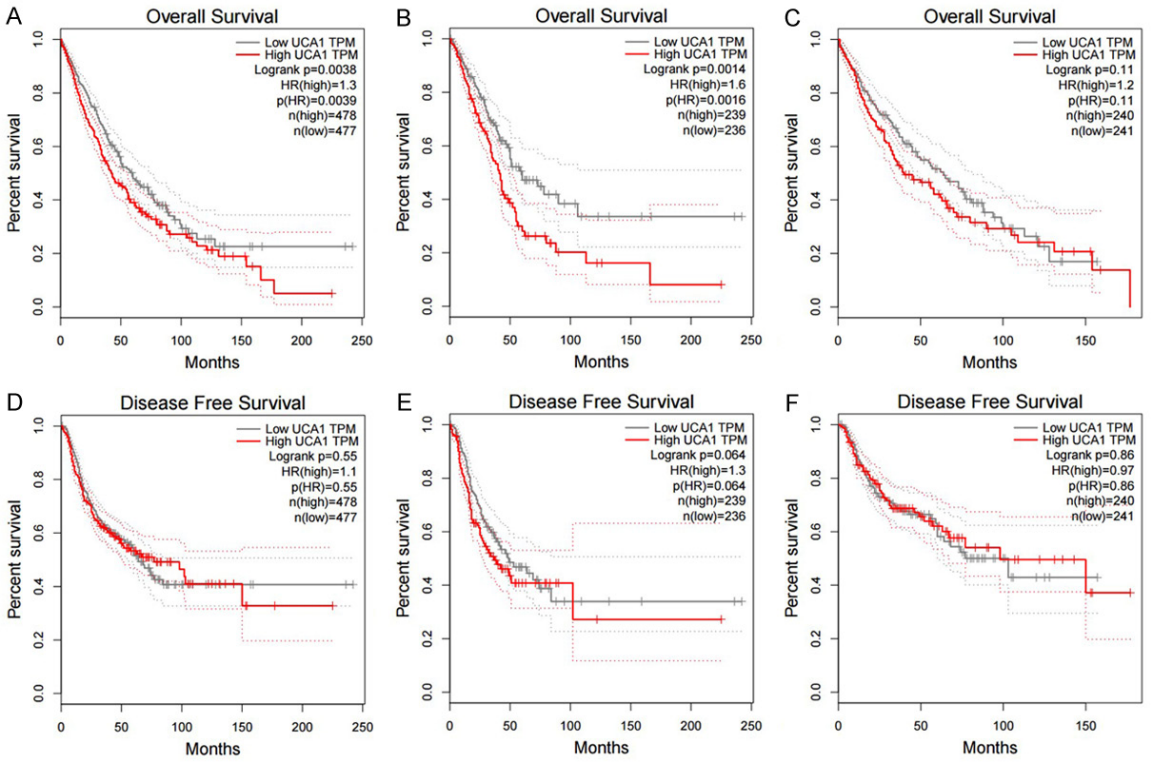

Figure 4. The survival curves of UCA1 based on the overall survival (OS) and disease-free survival (DFS) which are provided by GEPIA. A. The OS survival curve of UCA1 in NSCLC (logrank P=0.0039); B. The OS survival curve of UCA1 in LUAD (logrank P=0.0016); C. The OS survival curve of UCA1 in LUSC; D. The DFS survival curve of UCA1 in NSCLC; E. The DFS survival curve of UCA1 in LUAD; F. The DFS survival curve of UCA1 in LUSC. The cut-off of high/low expression group was the median transcripts per million reads (TPM) value. Red lines represent a high level of UCA1. Gray lines represent a low level of UCA1.

Genetic alterations of UCA1 in NSCLC from cBioPortal

The cBioPortal for Cancer Genomics provides visualization, analysis and download of large-scale cancer genomics data sets (http://www.cbioportal.org) [31-33]. Genetic alterations of UCA1 in NSCLC from different studies were collected from cBioPortal. Figures of OncoPrints concerning the UCA1 distribution and ratio of UCA1 genetic alteration types (amplification and deep deletion) were obtained from the website.

Effect of UCA1 siRNAs on cell caspase activity and apoptosis of LUAD cells

The LUAD cell line A549 was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured as recommended by the protocol.

Three UCA1 siRNAs were chemically synthesized by Gene Chem (Shanghai, China) and these three siRNAs were put together to form a pool for the knock-down experiments. The sequences of these three siRNAs were as following: UCA1-1, 5'-CCACCTGTAGAGAAGACAAA-3'; UCA1-2, 5'-GAAGAGTAGAAG ACAGGT-3'; UCA1-3, 5'-GCCTGGACAAGAACAGT-3', respectively. The siRNA transfection was performed with 24-well plates to test the transfection efficiency using CombiMAGnetofection (OZ BIOSCIENCES, Marseille cedex 9 France). After being transfected for 24, 48, 72 h and 96 h, the cells were collected to RT-qPCR and other analyses.

For the cell biological function assays, 2500 cells were prepared in 96-well plates and cultured overnight, then transfected with UCA1 siRNAs and corresponding controls. The cells were incubated again for another 24, 48, 72 h and 96 h, respectively for the following various biological function tests. The cell proliferation and viability were detected by the MTS and CellTiter-Blue assays. The status of caspase-3/7 activity was assessed by the Homogeneous Caspase-3/7 Assay. To verify the effect of UCA1 siRNAs on cell growth and apopto-

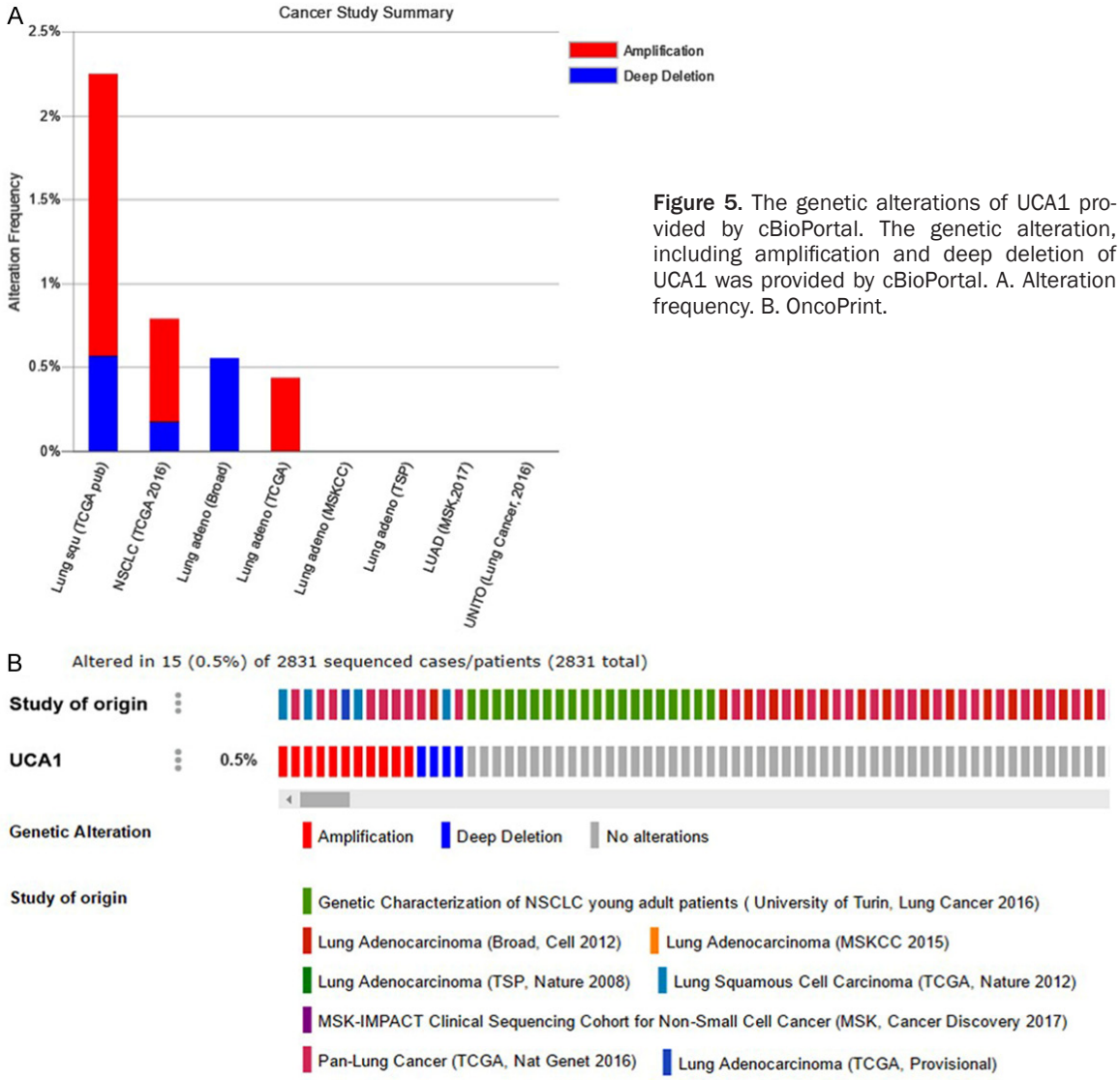

Figure 5. The genetic alterations of UCA1 provided by cBioPortal. The genetic alteration, including amplification and deep deletion of UCA1 was provided by cBioPortal. A. Alteration frequency. B. OncoPrint.

sis, Hoechst 33342/propidium iodide (PI) double staining was performed, and the cells were observed under a fluorescence microscope. All the above in vitro tests were conducted as described previously [34-39].

Statistical analysis

Besides those data provided by Oncomine, GEPIA and cBioPortal, the rest of the experimental data were analyzed by using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD. The ANOVA test was selected to examine the differences of the inhibitory ratios for cell growth or inducing ratios for cell caspase activity and apoptosis among the various groups. GraphPad Prism 5.0 was applied to draw all graphs. Two-sided $P<0.05$ was considered to be statistically significant.

Results

UCA1 expression in NSCLC tissues based on data from Oncomine and GEPIA

From Oncomine, two studies ("Hou Lung" and "Okayama Lung") provided the UCA1 expression data in different subtypes of NSCLC and non-cancer controls as detected by the Human Genome U133 Plus 2.0 Array. In the "Hou Lung" study, the number of cases of LUAD, LUSC, large cell lung carcinoma (LCC) and non-tumorous were 45, 27, 19 and 65, respectively

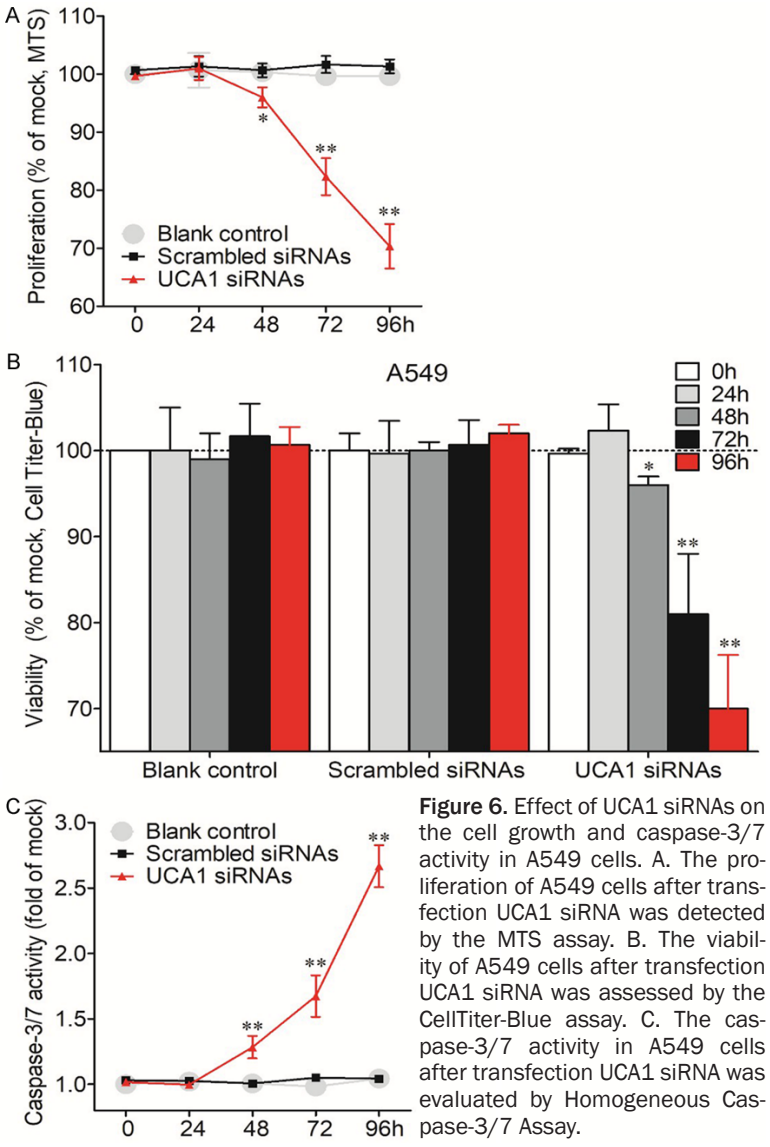

Figure 6. Effect of UCA1 siRNAs on the cell growth and caspase-3/7 activity in A549 cells. A. The proliferation of A549 cells after transfection UCA1 siRNA was detected by the MTS assay. B. The viability of A549 cells after transfection UCA1 siRNA was assessed by the CellTiter-Blue assay. C. The caspase-3/7 activity in A549 cells after transfection UCA1 siRNA was evaluated by Homogeneous Caspase-3/7 Assay.

(**Figure 1**). In the "Okayama Lung" study, there were 226 LUAD cases and 20 non-cancerous lung cases (**Figure 2**). After synthetically analyzing the data, we found an increasing trend for UCA1 expression in NSCLC tissues, as compared to that in non-cancerous lung tissues, especially in LUAD. However, the difference was not statistically significant. To further explore the expression level of UCA1 in NSCLC tissues, we sought the RNA-sequencing data calculated by GEPIA, which had been provided by TCGA and GTEx. The database uses log2 (TPM + 1) for its log-scale. Similarly, the UCA1 levels were up-regulated both in LUAD (T=483) and LUSC (T=486), as compared to that in non-cancer controls (non-LUAD: N=347, non-LUSC: N=338, **Figure 3A**). When LUAD and LUSC were placed together into NSCLC, the median level of UCA1 was 0.56, eight times higher than that in non-cancerous lung controls (**Figure 3B**). Hence, the over-expression trend of UCA1 could be found on both microarray and RNA-sequencing data. Then, we tried to analyze the potential function of UCA1 in the progression of NSCLC but found no obvious relationship between the UCA1 level and the clinical stages in NSCLC (**Figure 3C**), LUAD (**Figure 3D**) or LUSC (**Figure 3E**). Next, the survival analysis of UCA1 was calculated using the GEPIA database in NSCLC, LUAD and LUSC. The survival indicators were overall survival (OS) and disease-free survival (DFS). The cut-off of the high/low expression group was the median transcripts per million reads (TPM) value. In NSCLC, a higher level of UCA1 pointed to a poorer overall survival, with the HR being 1.3 (logrank P=0.0039, **Figure 4A**). In LUAD, the poorer prognostic role of UCA1 expression was more apparent considering the OS. The HR was 1.6, which further confirmed that UCA1 may be a risky factor for the poor outcome of LUAD (log rank P=0.0016, **Figure 4B**). In LUSC, however, no significant statistical significance was noted between UCA1 and OS (**Figure 4C**). The UCA1 level was not closely related to the DFS of NSCLC, LUAD or LUSC (**Figure 4D-F**).

Genetic alterations of UCA1 in NSCLC from cBioPortal

Genetic alterations of UCA1 in NSCLC were collected from cBioPortal, which provided various studies with the genetic alteration, including Genetic characterization of NSCLC young adult patients (University of Turin, Lung cancer 2016), Lung adenocarcinoma (Broad, Cell 2012), Lung adenocarcinoma (MSKCC 2015), Lung adenocarcinoma (TSP, Nature 2008), MSK-IMPACT clinical sequencing cohort for non-small cell

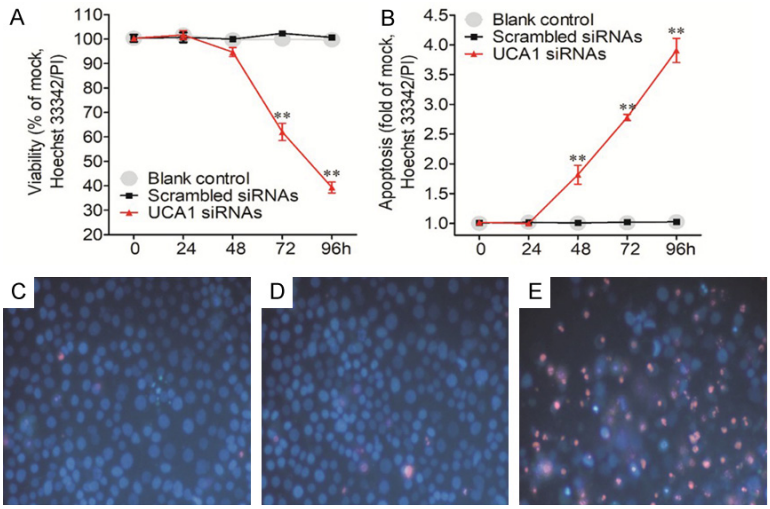

Figure 7. Validation of the influence of UCA1 siRNAs on the cell growth and apoptosis in A549 cells. (A) The viability of A549 cells by the Hoechst 33342/propidium iodide (PI) assay; (B) The apoptosis inducing effect of A549 cells by the Hoechst 33342/propidium iodide (PI) assay; Images of A549 cells incubated for 96 h, (C) Blank controls, (D) Scrambled siRNA controls, and (E) UCA1 siRNAs.

cancer (MSK, Cancer Discovery 2017), Pan-lung cancer (TCGA, Nat Genet 2016), etc. Two genetic alterations, amplification and deep deletion, were observed in UCA1 as provided by cBioPortal. **Figure 5A** displays the two alterations' frequencies in each study; **Figure 5B** shows the total frequency (0.5%) of alteration in all studies.

Effect of UCA1 siRNAs on cell caspase activity and apoptosis of LUAD cells

As the prognostic effect of UCA1 expression was more potent in LUAD than in LUSC, we then validated the function of UCA1 siRNAs on the biological reaction of LUAD cells *in vitro*. The A549 cell line was taken as an example for LUAD and after UCA1 siRNAs were transfected, different experiments were carried out to detect the variation of cell growth, caspase-3/7 activity and apoptosis. Both MTS and Cell Titer-blue assays showed the accordant inhibitory effect of UCA1 siRNAs on cell growth. From 48 h post transfection of UCA1 siRNAs, the growth speed was decreased for the A549 cells. At 96 h post transfection, the cell growth inhibitory rate reached 30% (**Figure 6A**, **6B**), which was accompanied by the increase of caspase-3/7 activity (**Figure 6C**). The caspase-3/7 activity was more than 2.5 times the blank and scrambled siRNA controls. To further verify the above finding, the cell growth, cell necrosis and apoptosis were tested by another independent approach, which focused on the morphological change of cells and could be observed under microscope. This Hoechst 33342/PI double staining confirmed the aforementioned data, which suggested that UCA1 may play a substantial part in the cell growth and cell apoptosis in LUAD cells (**Figure 7**).

Discussion

Urothelial cancer associated 1 (UCA1), a member of lncRNAs, was first discovered in human bladder cancer. It is found on human chromosome 19p13.12 and consists of three exons and two introns [6]. In some studies, UCA1 has been reported to be more over-expressed in NSCLC tissues than in normal lung tissues [27-29]. However, the clinical function and the mechanism of UCA1 in NSCLC remains incompletely understood. In this study, we verified that UCA1 was indeed significantly up-regulated in NSCLC after composite analysis the UCA1 expression in Oncomine, GEPIA and cBioPortal. Calculated by GEPIA, higher level of UCA1 in LUAD could lead to lower patient survival rates. The amplification and deep deletion were the main types of genetic alteration of UCA1 in NSCLC, which was provided by cBioPortal. We also performed in vitro experiments to explore the function of UCA1 in tumor proliferation and apoptosis. After transfection of UCA1 siRNA in the A549 cell line, the experiment's results showed that UCA1 could induce caspase-3/7 activity and apoptosis in A549 cells.

UCA1 was over-expressed in some lung cancer cell lines, including A549, H517, H446, H4006, H460, H1299 and H1650, as compared to cultured human lung epithelial cells (BEAS-2B), normal embryonic lung WI-38, and HEL-1 cells [27, 29] which was consistent with our previous detection (data not shown). As for the expression level of UCA1 in NSCLC tissues, two researches published the concordant up-regu-

lation of UCA1 with 112 [27] and 60 [28] pairs cases of NSCLC, respectively, which was detected by a real-time quantitative polymerase chain reaction (RT-qPCR). Here in our study, the up-regulating trend of UCA1 could also be observed in the data of some other detection methods: microarray and RNA-sequencing. The findings in our paper, together with those from earlier studies [27, 28] and our in-house RT-qPCR discovery (data not shown), verified the oncogenic role of UCA1 in the occurrence of NSCLC. Significantly, UCA1 also has the potential to be used as a non-invasive biomarker for the early screening of NSCLC, as it was reported that the circulating UCA1 level was also markedly up-regulated in NSCLC patients, with the AUC being 0.886 [28]. However, this diagnostic role was based on a single cohort with a small size of patients, so it remains to be validated with a larger sample size.

In addition to the role of UCA1 in the tumorigenesis of NSCLC, UCA1 has also been reported to play an essential part in the development of NSCLC. For instance, over-expression of UCA1 was closely related to histological grade and the status of lymph node metastasis [28]. UCA1 over-expression could cause an apparently poorer outcome of NSCLC patients, and multivariate analysis found that UCA1 could act as an independent risk factor of the prognosis of NSCLC [27]. A meta-analysis summarized all available studies concerning the prognostic role of UCA1 in NSCLC with three studies being involved [27, 28, 40]. Indeed, the result confirmed the risky role of UCA1 in the prognosis prediction ability of NSCLC, with the HR of 1.49 (95% CI: 1.16-1.90) [41]. This is in line with the current finding based on RNA-sequencing data with a large sample size of 955 patients, which led to the HR of 1.3. More importantly, the HR became more apparent in the subtype of LUAD, which reached 1.6. The current finding, with previous reports [41], further unveil the vital role of UCA1 in the progression and survival of LUAD, but not LUSC. The etiology of this difference still needs further investigation.

Due to the potent influence of UCA1 on the survival of LUAD, we then performed in vitro experiments to investigate the relevant biological function of UCA1 on LUAD A549 cells. Analogous cell growth inhibitory effect could be noted by UCA1 siRNAs in the current study, as compared to previous reports by other groups [27, 29]. Interestingly, we for the first time, found that the UCA1 siRNAs could strikingly increase the caspase-3/7 activity, which are executioner proteins of apoptosis in A549 cells. This effect on caspase-3 could also be found in other cancer types, such as cholangiocarcinoma [42], cervical cancer [14], prostate cancer [43] and gastric cancer [44]. Hence, UCA1 might play similar roles in different cancers to restrain the caspase-3/7 and enhance cell growth.

A relationship between UCA1 and EGFR mutation has also been reported. The UCA1 level was notably up-regulated in lung cancer cells and patients with acquired resistance to EGFR-TKIs. UCA1 silencing rescued gefitinib sensitivity in those acquired resistant cells which had non-T790M mutations. UCA1 may prompt non-T790M acquired resistance to EGFR-TKIs [40]. The molecular mechanism of UCA1 in NSCLC could also rely on the ceRNA axis. Two ceRNA axes of UCA1 in lung cancer have been clarified. One is UCA1/miR-144/Pre-B cell leukemia homeobox 3 (PBX3) [29], the other one is UCA1/miR-193-3p/ERBB4 [27]. Other axes still need to be explored for UCA1 in NSCLC.

To sum up, lncRNA UCA1 might play a substantial role in the occurrence and development of NSCLC, especially in LUAD patients, which is partly due to its effect on caspase-3/7 activity suppression. Additional potential molecular mechanisms of UCA1 in NSCLC require further assessment.

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Disclosure of conflict of interest

None.

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