Long Noncoding RNA LUCAT1 Promotes Multiple Myeloma Cell Growth by Regulating the TGF-β Signaling Pathway

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Abstract
Objective: Long noncoding RNAs (lncRNAs) are potential biomarkers for cancers. Nevertheless, the ability of long noncoding RNA lung cancer-associated transcript 1 in patients with multiple myeloma remains unknown. The purpose of this current study was to figure out its function in multiple myeloma. Methods: Firstly, the expression of long noncoding RNA lung cancer-associated transcript 1 in cancer or normal tissues and serum from patients with multiple myeloma and normal donors was detected. Secondly, the expression of long noncoding RNA lung cancer-associated transcript 1 was overexpressed or silenced in U266 and H929 cells, respectively to detect changes of proliferation and apoptosis in multiple myeloma in vitro. Subsequently, the expression of transforming growth factor-β signaling pathway-related proteins was detected by western blot analysis. Finally, the effect of long noncoding RNA lung cancer-associated transcript 1 on the growth of multiple myeloma cells in vivo was evaluated by tumor xenograft in nude mice. Results: Long noncoding RNA lung cancer-associated transcript 1 was increased in cancer tissues and serum of patients with multiple myeloma as well as multiple myeloma cells, which was correlated with dismal prognosis of patients with multiple myeloma. Overexpression of long noncoding RNA lung cancer-associated transcript 1 promoted the activity of U266 and H929 cells, while inhibition of long noncoding RNA lung cancer-associated transcript 1 suppressed the activity of U266 and H929 cells. In addition, long noncoding RNA lung cancer-associated transcript 1 was found to promote activation of the transforming growth factor-β signaling pathway. Furthermore, long noncoding RNA lung cancer-associated transcript 1 knockdown restricted the growth of multiple myeloma cells in vivo. Conclusion: This study suggests that suppression of long noncoding RNA lung cancer-associated transcript 1 inhibits the activation of transforming growth factor-β signaling pathway, thereby inhibiting the growth of multiple myeloma cells.

Keywords
multiple myeloma, lncRNA LUCAT1, TGF-β signaling pathway, proliferation, cell cycle arrest, apoptosis, tumor formation

Abbreviations
β2-MG, β2-microglobulin; ALB, albumin; CCK-8, cell counting kit-8; ccRCC, clear cell renal cell carcinoma; cDNA, complementary DNA; ceRNA, competing endogenous RNA; FITC, fluorescein isothiocyanate; ISS, International Staging System; lncRNAs, long noncoding RNAs; LUCAT1, lung cancer-associated transcript 1; MM, multiple myeloma; nPCs, normal plasma cells; PI, propidium iodide; RT-qPCR, reverse transcription quantitative polymerase chain reaction; siRNA, small interfering RNA; TGF-β, transforming growth factor-β

Introduction
Multiple myeloma (MM) is a type of plasma cell tumor featured by immunodeficiency, renal disease, overproduction of monoclonal immunoglobulins, and osteolytic bone lesions. The early clinical symptoms in patients with MM consist of anemia, musculoskeletal pain, as well as susceptibility to...
infection, but at late stage, there exist pancytopenia, fracture, renal insufficiency, along with some neurological signs.\(^2\) The epidemiology of MM is widely investigated, and older age, male sex, black race, positive family history, and genetic factors are regarded as risk factors for this disease.\(^3\) Multimodality approaches such as traditional chemotherapy and stem cell transplantation are applied in MM therapy.\(^4\) The survival rate of patients with MM varies significantly, ranging from precious few weeks to 10 years above, and the 5-year survival rate is approximately 40%.\(^5\) In view of this, efficacious approaches could enhance the probability of timely therapy and improve the prognosis for patients with MM.

Long noncoding RNAs (lncRNAs) are abnormally expressed in varying kinds of cancers, and also to be implicated in either oncogenic or tumor-suppressive properties.\(^6\) Evidence has demonstrated that lncRNAs are implicated in MM, and also serve as vital biomarkers.\(^7\) For instance, there are articles demonstrating that the upregulated lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is of great importance in pathogenesis of MM, \(^8,9\) while downregulated lncRNA KIAA0459 is essential for myeloma.\(^10\) Long noncoding RNA lung cancer associated transcript 1 (LUCAT1), the nickname of smoke and cancer associated lncRNA 1, has been initially described to participate in smoking-associated lung cancer.\(^11\) Long noncoding RNA LUCAT1 has been revealed as a stimulative gene in clear cell renal cell carcinoma (ccRCC) and esophageal squamous cell carcinoma.\(^12,13\) Nevertheless, the capability of lncRNA LUCAT1 in MM is still unexplored. Transforming growth factor-\(\beta\) (TGF-\(\beta\)) appears to be elevated in bone lesions of MM, which might play a role in bone formation injured by MM.\(^14\) Transforming growth factor-\(\beta\) is able to affect myeloma progression via its functions on the tumor microenvironment by exacerbating lytic bone disease, impairing normal immune responses, as well as stimulating angiogenesis.\(^15\) The data from a previous study has suggested that TGF-\(\beta\) signaling pathway blockade decreased tumor burden and alleviated the tumor microenvironment so as to hamper the myeloma progression.\(^16\) From the aforementioned, we speculated that lncRNA LUCAT1 exerted its function in MM by modulating the TGF-\(\beta\) signaling pathway.

**Materials and Methods**

**Clinical Sample Collection**

The tumor tissues from 47 patients with MM and the bone marrow tissues from 13 healthy donors were collected from the Department of Pathology of the Second Hospital of Shanxi Medical University. Purification and isolation of primary CD138\(^+\) cells in bone marrow were performed according to the procedures reported previously.\(^17\) Besides, fasting blood was obtained from all patients for serum extraction. The patients combined with other malignant diseases or with extramedullary myeloma were excluded. The diagnostic criteria, disease status, and response to treatment of myeloma are based on the standards of the International Myeloma Working Group. This study was ratified and supervised by the ethics committee of the Second Hospital of Shanxi Medical University (No. 2013-05024). All patients with MM and healthy donors provided the informed consent.

**Enzyme-Linked Immunosorbent Assay**

An automatic biochemical analyzer was applied to measure levels of albumin (ALB) and \(\beta\)-2-microglobulin (\(\beta\)-2-MG) in the serum of participants.

**Cell Culture and Grouping**

Human MM cell lines (MM.1S, KM3, RPMI-8226, U266, and H929) were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Normal plasma cells (nPCs) and MM cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco) with 10% fetal bovine serum at 37°C with 5% CO\(_2\).

Subsequently, the well-grown U266 and H929 cells were harvested, and the constructed pcDNA3.1 LUCAT1 and the corresponding empty vector pcDNA3.1 were transfected into the cells. Similarly, the well-grown U266 and H929 cells were taken, and the constructed small interfering RNA (siRNA)-LUCAT1 and the corresponding scramble siRNA were transfected into the cells. All operations were strictly performed upon the instructions of Lipofectamine 2000 reagent. Forty-eight hours post transfection, LUCAT1 expression in the cells was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR), and the transfection efficiency was verified.

**Reverse Transcription Quantitative Polymerase Chain Reaction**

Total RNA from tissues and cells was extracted using TRizol (Thermo Fisher Scientific). Subsequently, the first complementary DNA (cDNA) strand was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). After 10-fold dilution, the cDNA was used for RT-qPCR experiments by using a SYBR Green PCR Kit (Takara). Primer sequence: LUCAT1: reverse (R) 5'–AGCTCACCTCCCCGGTTACCG-3'; forward (F) 5'–CGTGAACCCGGAGGGCT-3'.

**Cell Counting Kit-8 Assay**

In this experiment, cell counting kit-8 (CCK-8) and colony formation assays were used to detect cell proliferation and viability. For CCK-8 assay, all operations were strictly performed in the light of the requirements of CCK-8 kits (Dojindo). Briefly, cells were seeded in 96-well plates at a density of \(5 \times 10^3\) cells/each well and cultured for 24 hours, 48 hours, or 72 hours. The cells were maintained for another 1 hour at 37°C after the addition of 10 \(\mu\)L CCK-8. The optical density value of each well at 450 nm was recorded using a
microplate reader (Bio-Rad). For colony formation assay, the experimental procedures were performed according to a previous report. In brief, cells were plated in a 6-well plate at 300 cells/well. After 2 weeks of conventional culture, the formed colonies (> 50 cells/colony) were fixed with methanol, stained with crystal violet, and counted under a microscope.

**Flow Cytometry**

Flow cytometry was implemented to detect the cell cycle distribution and apoptosis of U266 and H929 cells. The experimental procedures were performed referring to a previous report. For the cell cycle assessment, the transfected cells of each group were fixed overnight at 4 °C. After that, cells were stained with propidium iodide (PI) for 15 minutes. The cell distribution in G1, S, and G2 phases was evaluated by flow cytometry. For the detection of apoptosis, Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime) was used to detect apoptosis. After seeding the cells at 2 × 10^5 cells/well in a 6-well plate for 24 hours 10 μL Annexin V-FITC and PI solution were added. After 15 minutes, apoptotic cells were measured by a flow cytometer (Beckman Coulter) and analyzed using FACScan.

**Western Blot Analysis**

The extent of TGF-β signaling pathway-related protein phosphorylation in U266 cells and H929 cells were determined by western blot analysis. The experimental procedures were performed according to a previous report. In short, radio immunoprecipitation assay lysis buffer was applied to extract the primary antibody and with the corresponding secondary antibody at 37 °C for 2 hours. Primary antibodies to TGF-β1 (1: 5000, ab92486), pSmad2/3/4/5/8 (1: 1000, ab53100), Smad2 (1: 2000, ab63576) and secondary horseradish peroxidase-labeled goat anti-rabbit antibody (1: 10000, ab7090, Abcam) were utilized. All the antibodies were purchased from Abcam Inc. Blots were examined by an enhanced chemiluminescence detection kits and analyzed using ImageJ (National Institutes of Health).

**Tumor Xenograft in Nude Mice**

Four-week-old female BALB/c nude mice were acquired from the Shanghai Experimental Animal Center of Chinese Academy of Sciences. H929 cells post transfection were subcutaneously injected into the posterior flank of nude mice. The nude mice were euthanized 35 days later, and the tumors tissues were harvested for further analysis. Subsequently, tumor volume was counted every week and tumor weight was then measured. Tumor volume was calculated based upon the following formula: tumor volume (mm³) = 0.5 × length × width². All animal experiments were performed with the approval of the Animal Care and Experiment Committee of the Second Hospital of Shanxi Medical University (No. 201806011). Significant efforts were made to minimize the number of animals and their pain.

**Immunohistochemical Staining**

Mice were euthanized by pentobarbital sodium at 100 mg/kg, and tumor tissues were then extracted for immunohistochemical staining. The experimental procedures were performed in the light of a previous literature. Rabbit anti-human Ki67 (1: 500, ab15580, Abcam) and goat anti-rabbit secondary antibody (1: 1000, ab7090, Abcam) were used in the experiment.

**Statistical Analysis**

All data were processed by SPSS version 21.0 statistical software (IBM). The data in normal distribution detected by Kolmogorov-Smirnov test were depicted in the form of mean ± standard deviation. The comparison between the 2 groups was analyzed by unpaired t test. Data among 3 or more groups were analyzed by using 1-way or 2-way analysis of variance, followed by Tukey’s multiple comparisons test. Pearson correlation test was used to analyze the correlation between LUCAT1 and serum ALB and β2-MG levels. P value less than .05 showed significant difference.

**Results**

**Long Noncoding RNA LUCAT1 Is Upregulated in MM Samples and Cell Lines, and Is Associated With Poor Prognosis of Patients With MM**

We firstly tested the expression of IncRNA LUCAT1 in MM tissues and serum from 47 patients and the bone marrow tissues from 13 healthy donors. The results suggested that IncRNA LUCAT1 was upregulated in tissues and serum of patients with MM (Figure 1A). Correspondingly, IncRNA LUCAT1 expression was correlated to the International Staging System (ISS) stage of patients with MM, and IncRNA LUCAT1 expression was higher in patients with MM with more advanced ISS stage (Figure 1B). Subsequently, we found that IncRNA LUCAT1 expression in MM cells was higher than that of nPCs (Figure 1C), and the LUCAT1 expression was more pronounced in U266 and H929 cell lines, which were selected for the following assays. We then examined the protein levels of ALB and β2-MG in the serum of 47 patients with MM and 13 healthy donors. We observed that the levels of ALB in serum of patients with MM were significantly lower than those in healthy donors, while the levels of β2-MG were remarkably higher (Figure 1D, E). We further used Pearson correlation test to analyze the association between LUCAT1 expression and
ALB and $\beta_2$-MG protein levels. It was noted that LUCAT1 expression was negatively correlated with ALB and positively correlated with $\beta_2$-MG levels (Figure 1F, G). We subsequently divided patients with MM into high expression and low expression groups based on LUCAT1 expression. Kaplan-Meier analysis revealed that patients with MM with higher expression of LUCAT1 had shorter 5-year survival (Figure 1H).

Long Noncoding RNA LUCAT1 Overexpression Enhances Proliferation and Inhibits Cell Cycle Arrest and Apoptosis of MM Cells in Vitro

Next, we induced U266 and H929 cell lines stably overexpressing lncRNA LUCAT1 (Figure 2A). Cell Counting Kits-8 were utilized to detect MM cell viability, and it was revealed that overexpression of lncRNA LUCAT1 promoted U266 and H929 cell viability (Figure 2B). The results of colony formation assays further revealed that the number of colonies formed by U266 and H929 cells increased significantly after LUCAT1 overexpression (Figure 2C). Subsequently, we performed flow cytometry to detect the cell cycle distribution of U266 cells and found that lncRNA LUCAT1 induced an elevation in the number of U266 and H929 cells in S phase and suppressed cell apoptosis (Figure 2D, E).

Long Noncoding RNA LUCAT1 Downregulation Limits Proliferation and Boosts Cell Cycle Arrest and Apoptosis of MM in Vitro

To further verify the capabilities of lncRNA LUCAT1 knockdown in MM, we developed H929 and U266 cells that stably interfering with lncRNA LUCAT1 expression (Figure 3A). The results of CCK-8 and colony formation assays showed that downregulation of lncRNA LUCAT1 inhibited the viability and colony formation abilities of H929 and U266 cells (Figure 3B, C). Additionally, downregulated lncRNA LUCAT1 also promoted the cell cycle arrest of H929 and U266 cells in the G0/G1 phase, thereby promoting the apoptosis of H929 and U266 cells (Figure 3D, E).

Long Noncoding RNA LUCAT1 Promotes MM Growth by Activating the TGF-β Signaling Pathway

Next, we tested the activation of the TGF-β signaling pathway in H929 and U266 cells, and found that overexpression of lncRNA LUCAT1 elevated the extent of Smad2 phosphorylation and expression of TGF-β1 in U266 cells. On the contrary, inhibition of lncRNA LUCAT1 in H929 cells had the opposite tendency (Figure 4).
Suppression of lncRNA LUCAT1 Inhibits MM Growth in Vivo

With the aim to verify the effect of lncRNA LUCAT1 on the growth of MM cells in vivo, tumor xenograft in nude mice was carried out. The results indicated that inhibition of LUCAT1 restricted the growth rate and tumor weight of xenografts in vivo (Figure 5A, B). Moreover, the number of Ki67 positive cells in the tumors was reduced after lncRNA LUCAT1 knockdown (Figure 5C).

Discussion

In recent years, great advances have been witnessed in the understanding of the occurrence and progression of MM.21 More recently, the abnormal expression of lncRNAs was found in patients with MM, implying their potentials as biomarkers for MM therapy.8 Because of the significant differences in the survival of patients with MM, the stratification of prognosis and treatment for patients are important for the improvement of prognosis of the patients. Therefore, the molecular mechanisms of lncRNAs related to the MM development need further study.

One of the obtained results implied that lncRNA LUCAT1 was upregulated in MM tissues and cells, which was also correlated to poor prognosis. We also found that the expression of lncRNA LUCAT1 was positively related to ISS and β2-MG, while negatively associated with the ALB levels in the serum, which are both powerful predictors of survival for patients with MM.22 These results implied that lncRNA LUCAT1 could assist clinicians to monitor the condition and severity of disease of patients with MM. Similar to our findings, lncRNA LUCAT1 upregulation has been indicated to be positively related to the poor survival rate of patients with liver cancer.23 Another study has demonstrated that lncRNA LUCAT1 was overexpressed in ccRCC tissues, which was associated with progression free survival and shorter overall survival of ccRCC.24 Sun et al have stated that lncRNA LUCAT1 is overexpressed in non-small cell lung cancer tissues, and its high expression is associated with positive lymph node metastasis, high tumor, node, metastases staging as well as poor prognosis.25 In addition, our CCK-8, colony formation assays, and flow cytometry suggested that overexpressed lncRNA LUCAT1 resulted in promoted activity of U266 cells, while inhibition of lncRNA LUCAT1 presented an opposite trend.
Consistently, the cell proliferation rate and the number of cell clones was remarkably reduced, yet the apoptosis rate was distinctly enhanced in ovarian cancer cells with lncRNA LUCAT1 silencing. Moreover, suppression of lncRNA LUCAT1 inhibited the growth of MM cells in vivo. Intriguingly, an article has demonstrated that knockdown of lncRNA LUCAT1 restricted epithelial-mesenchymal transition process of hepatoblastoma cells, implying the stimulative effect of lncRNA LUCAT1 in hepatoblastoma. Evidence has pointed out that lncRNA LUCAT1 expression was enhanced in the tissues and cells of glioma, and depleted lncRNA LUCAT1 hindered growth of glioma cells in vitro. Furthermore, silencing of lncRNA LUCAT1 has also been found to restrict cell progression in vitro, and suppressed tumorigenesis of ccRCC cells in vivo. More recently, LUCAT1 has been suggested to promote the progression of choroidal melanoma through a competing endogenous RNA (ceRNA) mechanism of action. Therefore, we are considering to carry out further experiments to evaluate whether LUCAT1 functions as a ceRNA in MM.

Besides, our study also indicated that lncRNA LUCAT1 induced the activation of the TGF-β signaling pathway, as evidenced by promoted Smad2 phosphorylation level and the expression of TGF-β1. Transforming growth factor-β has been indicated to activate downstream Smad2 and Smad3 to promote the formation of their complex and their phosphorylation. Moreover, SB431542, a TGF-β1 inhibitor, diminished cell invasion in MM cells, which was mediated via the TGF-β1/Smad2/matrix metallopeptidase 3 signaling pathway. A prior study has elucidated that some lncRNAs are involved in some classical pathways including TGF-β pathway. It is reported that knockdown of lncRNA MALAT1 decreased TGF-β1 secretion and LTBP3 transcription. Zhang et al supported that TGF-β expression is highly expressed in MM samples, which has a positive relation with lncRNA urothelial carcinoma antigen 1 (UCA1). The suppressive effect was neutralized when overexpressing TGF-β in MM cells introduced with lncRNA UCA1 short hairpin RNA. Patients with myeloma have been found to generate higher levels of TGF-β relative to nPCs, resulting in the immune dysfunction in patients with MM. Meanwhile, the finding of this current study also revealed that lncRNA LUCAT1 impaired the activation of the TGF-β signaling pathway, thereby suppressing the growth of MM cells.

Suppression of the TGF-β signaling pathway contributed to an increase of bone formation and an inhibition of MM cell growth. Takeuchi et al stated that TGF-β inhibition may ameliorate tumor growth and improved MM-related clinical outcomes. Therefore, we are considering to carry out further experiments to evaluate whether lncRNA LUCAT1 functions as a ceRNA in MM.

**Figure 3.** Long noncoding RNA (lncRNA) LUCAT1 downregulation restricts proliferation and promotes cell cycle arrest and apoptosis of MM in vitro. A, LncRNA LUCAT1 is downregulated in H929 and U266 cells after transfection of LUCAT1 siRNA. B, LncRNA LUCAT1 knockdown inhibits cell viability in MM cells by CCK-8 assay. C, LncRNA LUCAT1 knockdown reduces the number of colonies by colony formation assay. D, LncRNA LUCAT1 knockdown induces cell cycle arrest at G0/G1 phase in H929 and U266 cells by flow cytometry. E, LncRNA LUCAT1 knockdown increases the percentage of apoptotic cells. Data are expressed as mean ± standard deviation, representative of 3 independent experiments. Two-way ANOVA and Tukey multiple comparisons test or unpaired t test was used to determine statistical significance. *P < .05. ANOVA indicates analysis of variance; CCK-8, cell counting kit-8; LUCAT1, lung cancer-associated transcript 1; MM, multiple myeloma; siRNA, small interfering RNA.
Figure 4. Long noncoding RNA (LncRNA) LUCAT1 promotes MM growth by inducing the TGF-β signaling pathway, as determined by western blot analysis. Data are expressed as mean ± standard deviation, representative of 3 independent experiments. Two-way ANOVA and Tukey multiple comparisons test was used to determine statistical significance. *P < .05. ANOVA indicates analysis of variance; LUCAT1, lung cancer-associated transcript 1; MM, multiple myeloma.

Figure 5. Long noncoding RNA (LncRNA) lung cancer-associated transcript 1 (LUCAT1) suppression restricts MM growth in vivo. A, LncRNA LUCAT1 knockdown reduces tumor volumes of nude mice. B, LncRNA LUCAT1 knockdown decreases tumor weights of nude mice. C, The relative expression of Ki67 in tumor tissues of nude mice were examined. Data are expressed as mean ± standard deviation, representative of 3 independent experiments. Two-way ANOVA and Tukey’s multiple comparisons test or unpaired t test was used to determine statistical significance. *P < .05. ANOVA indicates analysis of variance; MM, multiple myeloma.
features.14 Furthermore, a study has indicated that cell proliferation was suppressed in TGF-β-treated MM.1S cells, which was reversed with anti-E2F1 siRNA treatment.55

Collectively, this study indicates that suppressed IncRNA LUCAT1 blocked the activation of the TGF-β signaling pathway, thereby suppressing the growth of MM cells. In addition, IncRNA LUCAT1 might be a vital biomarker to assess prognosis in MM survivors.

Authors’ Note
All the data generated or analyzed during this study are included in this published article. Our study involving human samples was carried out under the approval of the Ethics Committee of the Second Hospital of Shanxi Medical University (No. 2013-05024). All patients with MM and healthy donors provided the informed consent. All animal experiments were performed with the approval of the Animal Care and Experiment Committee of the Second Hospital of Shanxi Medical University (No. 201806011). Significant efforts were made to minimize the number of animals and their pain.

Declaration of Conflicting Interests
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