Original Article

Long non-coding RNA LUCAT1 promotes cell proliferation and invasion in melanoma

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Abstract

Background: Melanoma is a serious malignant cancer with a low survival rate. On a global scale, breast cancer is the most frequent malignancy and leading cause of cancer death in women. Long non-coding RNAs (lncRNAs) can be used effectively as regulators and biomarkers in several cancers. Accordingly, the treatment plans of cancer patients could be made easier because of this. It has been reported that lncRNAs can play regulatory functions in various cancers, including melanoma. It is necessary to improve melanoma research programs and health policies, including in poor countries, around the world.

Objective: The function of lncRNA lung cancer associated transcript 1 (LUCAT1) in melanoma has still not been identified. In the present study, large-scale screening for the differentially expressed lncRNAs was performed by lncRNAs microarray and finding the relationship between LUCAT1 and stemness marker.

Methods and materials: LncRNA LUCAT1 expression was assessed in cancer tissues by *in situ* hybridization. Sphere-formation assay and colony-formation assay were used to detect cell self-renewal and proliferation, respectively. RNA pull-down and luciferase reporter assays were used to identify LUCAT1.

Results: Silenced LUCAT1 can reduce cell growth, migration and invasion, and promote cell apoptosis, of melanoma. Conversely, over-expressed LUCAT1 can promote the progression of melanoma cells.

Conclusions: The expression of LUCAT1 in melanoma cells was detected via quantitative real-time polymerase chain reaction (qRT-PCR) assay. We found that lncRNA LUCAT1 was significantly upregulated in melanoma cells. Then, we further searched the role of lncRNA LUCAT1 in melanoma. [*Ethiop. J. Health Dev.* 2020; 34(4):293-300]

Key words: LUCAT1, melanoma, QRT-PCR assay, transwell migration, health science

Introduction

Despite steady progress in research related to cancer and melanoma, there are still many challenges. Improving research in this area will allow us to lessen the threat caused by melanoma and decrease the mortality rate. Research should focus on early detection, tumor cell dormancy and therapy. A detailed understanding of tumor signaling causing melanoma will help us to develop specific therapies and drugs, and decrease the burden of tumors. The sole purpose of this research work is to use biotechnology as tool to diagnose highly prevalent diseases to benefit human health and society as a whole.

Acting as an aggressive skin cancer, melanoma is responsible for the loss of many lives (1). The occurrence of melanoma has been increasing and accounts for nearly 5% of all skin cancers (2). Because of the metastasis character of melanoma, the five-year survival rate is just 13% (3-4). Even though there have been significant improvements in the diagnosis of melanoma, the same cannot be said of treatment. Therefore, it is vital to conduct research to search for the inner mechanisms underlying melanoma.

Long non-coding RNAs (lncRNAs) have been reported to play crucial regulatory roles in the progression of various human diseases (5). In the case of cancer, this includes cell growth, apoptosis, migration and invasion (6). For example, LINC00880 (long intergenic non-protein coding RNA 880) can act as an oncogene in spinal cord ependymoma to activate cell growth and progress epithelial mesenchymal transition (EMT) by modulating the expression of calcium voltage-gated channel auxiliary subunit gamma 5 (CACNG5), a protein coding gene (7). And LINC00673 can promote the cell growth and migration of non-small cell lung cancer (NSCLC) cells by regulating MiR-150-5p

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expression (8). Lung cancer associated transcript 1 (LUCAT1) has been identified as acting as an oncogene in many cancers, such as NSCLC, esophageal squamous cell carcinoma, and clear cell renal cell carcinoma (9-11). However, the function of lncRNA LUCAT1 in melanoma has still not been identified. This study aims to explore its function in relation to melanoma.

LncRNAs, which are a type of RNA, contain more than 200 nucleotide sequences; these sequences do not have the ability to encode a protein or translate into protein. As noted above, lncRNAs have the ability to participate in cell proliferation, differentiation and apoptosis. Accordingly, they are capable of controlling various biological pathways. Research has shown that lncRNAs have the capability of upregulating and downregulating the progression of tumor cells. Studies have shown that LL22NC03-N64E9.1 is a class of RNA which is responsible for the progression and metastasis effect of cancerous cells on the lung's wall. As with previous examples, LL22NC03-N64E9.1 has a positive effect on the Wnt/B catenin signaling pathway, where interaction between catenin B and protein first starts the pathway and then it consistently activates signaling Junction. This pathway is different from the LL22NC03 in terms of the location of the cancer and the type of cancerous cell. Basically, LINC00210 is responsible for malignant phenotype of liver cancer.

Breast cancer is one type of cancer that can be initiated by the effect of lncRNA-Hh. LncRNA-Hh activates the hedgehog signaling pathway, which is involved in transmitting information to embryonic cells required for proper cell differentiation to promote the progression of breast cancer cells. Research is ongoing to understand the mechanism of LUCAT1 in breast cancer progression.

Recent studies have focused on some of the prognostic factors that have positive effects on the progression of carcinoma cells. LncRNA LUCAT1 is one type of adverse prognostic factor that is responsible for the progression of carcinoma cells. It is able to initiate hepatocellular carcinoma, acting like a signaling molecule. In this signaling pathway, LUCAT1 inhibits the function of Annexin A2 to initiate the tumorigenesis.

Research suggests that functions of LUCAT1 are linked with p21 and p57 expression, which are the cyclin-dependent kinase inhibitors (Kip2), (Waf1/Cip1/Sdi1) and (Kip1). LUCAT1 is epigenetically repressed p21 and p57 expression. Due to this terminate p21 and p57 expression system, LUCAT1 is able to take control of the development and cell proliferation of NSCLC.

Cell viability can be affected by the elimination of lncRNA LUCAT1. The function of LUCAT1 is dependent on the micro RNA signaling pathway. For example, by regulating the miR-612 pathway, LUCAT1 can initiate ovarian cancer and is responsible for the progression of cancerous cells. As noted above, the function of LUCAT1 in breast cancer is not well identified. LncRNA can function like micro RNA sponges, whereas competitive endogenous RNA (ceRNA) can also serve like micro RNA and lncRNA. These three class of RNA show a similar type of function and all are responsible for the tumorigenesis of hepatocellular carcinoma. LncRNA MIR31HG has a negative effect on carcinoma progression, modulating ST7L expression and inhibiting cancer cells' proliferation. This gene produces a lncRNA that acts as a host gene for MiR31 and sponge MiRNA575. In addition, the degradation of lung adenocarcinoma can be expected when NEAT1 (a class of lncRNA) is successfully engaged with mir-193a-3p. This kind of engagement site can be observed between LUCAT1 and micro-RNA5702. Research reveals that LUCAT1 is not closely linked with the progression of triple negative breast cancer (TNBC). But it does helps to develop tumorigenesis of TNBC by interacting with MiR5702. In the future, this junction (lncRNA–MiR5702) could be targeted as a break point for cancer to develop new therapeutic drugs.

LncRNA LUCAT1 is directly linked with clear cell renal cell carcinoma. LUCAT1 is the most common provoking gene that has a clear relationship with the development of esophageal cell carcinoma. In addition, LUCAT1 is associated with multiple myeloma, which is a class of tumor in plasma cells. In this type of tumorigenesis, the amount of transforming growth factor B (TGF-B) in the plasma is always above its normal level. TGF-B is one of the essential factors in the formation of bone that is damaged by multiple myeloma, and plays a crucial role on the proliferation of myeloma cells because of their activity in the site of the cancer. They simply increase lytic bone disease and block the body's own immune system to protect against cancer cells, thereby initiating angiogenesis. If the TGB-B signaling pathway is blocked for any reason, it will slow

down the progression of the myeloma. LUCAT1, with the help of miR-200c, can control osteosarcoma formation. As said earlier, LUCAT1 acts as a stimulant of the AKT pathway, which can regulate the progression of carcinoma. Research is still ongoing on the effect of the LUCAT1 gene on breast cancer progression, so the mechanism of the gene in breast cancer proliferation is still unclear. Sometimes, LUCAT1 can act like an inhibitory molecule in breast cancer development. With the help of MiR-7-5P, the LUCAT1 gene exhibits increased levels of SOX2 adsorption and this increases the response to SOX2 sensitivity, a transcription factor essential for maintaining self-renewal, and will inhibit the progression of breast cancer.

As discussed above, LUCAT1 regulates the differentiation and progression of clear cell renal cell carcinoma. Regulation is through the ATK signaling pathway. When the chemokine CXCL2 is released from the carcinoma cell then over-expression of the LUCAT1 gene occurs. Over-expression of the LUCAT1 gene will enhance the proliferation rate of carcinoma cells and arrest all nearby cells in the G1 phase of the cell cycle. In the present study, we explore the function of LUCAT1 in melanoma.

Materials and methods

Cell culture: Human melanoma cell samples (A2058, M21, A375, A-875) and human keratinocytes (HaCaT), available from ATCC (Rockville, Maryland, USA), were preserved in Dulbecco's Modified Eagle Medium (DMEM), which is used for cultivation of cell cultures (Invitrogen, Carlsbad, CA, USA). Cell culture was achieved with the application of 10% fetal bovine serum (FBS; Invitrogen) and 1% Pen/Strep mixture under standard condition of 5% CO_2 and 37ºC, with DMEM replaced every three days.

RNA extraction and quantitative real-time PCR (qRT-PCR): Total RNAs from culture cell samples were extracted by use of Invitrogen TRIzol reagent, a monophasic solution of phenol and guanidine isothiocyanate, for the reverse transcription in light of the manual (Thermo Fisher, Waltham, MA, USA). qPCR was conducted by employing SYBR green Supermix (Thermo Fisher). The change in gene expression was calculated with the comparative change-in-cycle method (ΔΔCt), normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or U6 expression.

Plasmid transfection: The synthesized short hairpin RNAs for LUCAT1 and negative control (NC) shRNAs were procured from Genepharma Company (Shanghai, China). In addition, pcDNA3.1/LUCAT1 and NC pcDNA3.1 vector (both Genepharma Company) were used for over-expressing LUCAT1. pcDNA 3.1 contains an ampicillin antibiotic resistance site, regions of the CMV promoter and SV40 promoter site. A target opens reading frame without any stop codon. pUC region along with F1 region. The pUC and the F1 region is the origin of replication site. There are few restricted sites present such as Nhe I, BahmH 1, Hind III and Kpn I. The 48 hour of plasmid transfection in the A2058 cell line was implemented in line with the instruction of kit Lipofectamine 2000 (Invitrogen).

Colony formation: A2058 cell lines at the log phase of growth were harvested and resuspended, then seeded into the 6-well, plated with a density of 500 cells per well. Cells were cultured for two weeks. After washing in modified Dulbecco's phosphate-buffered saline (PBS), cells were fixed using 4% paraformaldehyde, and then processed with 0.1% crystal violet staining solution. The clones were counted visually.

EdU staining assay: Using the 5-ethynyl-20-deoxyuridine (EdU) incorporation assay kit, cell proliferation was assessed by EdU staining assay, as per the user guide (Ribobio, Guangzhou, China). After 48 hours of transfection, each well of 96 well plates were filled with EdU medium diluent. Three hours later, cells were fixed and permeabilized for nuclear detection with DAPI (4',6-diamidino-2-phenylindole) dye. Stained cells were assayed under fluorescence microscope (Nikon, Tokyo, Japan).

Transwell migration and invasion assay: Migration assay for A2058 cells was performed with transwell chambers (Corning, Corning, NY, USA) of 8mm pore size. The lower chamber was added with DMEM containing 10% FBS. DMEM is the most broadly suitable medium for many adherent cell phenotypes among defined media for cell and tissue culture. DMEM is a modification of Basal Medium Eagle (BME) that contained 1,000 mg/l of glucose and was first reported

for culturing embryonic mouse cells. A further alteration with 4,500 mg/l glucose has proved to be optimal for the cultivation of certain cell types. A2058 cells in serum-free DMEM were put into the upper chamber. Chambers were cultivated at 37°C in 5% CO_2 for 48 hours. Then cells in the upper chamber were removed with cotton swabs, while those on the bottom surface were counted using 0.1% crystal violet dye and microscope. Invasion assay for A2058 cells was performed using transwell chambers coated with Matrigel (Clontech, Madison, WI, USA).

Flow cytometry apoptosis assay: After 48 hours of transfection, A2058 cells (2 × 105) were reaped and rinsed in pre-cooled PBS. Apoptotic cells were monitored using an Annexin V-FITC/PI apoptosis kit (Life Technologies, Carlsbad, CA, USA). Annexin V and propidium iodide (PI) were both purchased from BD Biosciences (Franklin Lakes, NJ, USA) and added to the binding buffer. After 15 minutes of treatment, cells were assayed applying the FACSCalibur flow cytometer (BD Biosciences).

TUNEL staining assay: Terminal deoxynucleotidyl-transferase (TdT) mediated dUTP nick-end labeling (TUNEL) staining assay was applied to measure the cell apoptosis level of A2058. Based on the protocol supplied with the *in situ* cell death detection kit (Roche, Basel, Switzerland), cultured A2058 cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. Following incubation in the TUNEL reaction mixture, cell nuclei were stained with DAPI after one hour. Samples were visualized using fluorescence microscopes.

TdT is an enzyme which tries to repair double stranded breaks. It searches for a break in the DNA strand and incorporates itself there and converts the nick end of the DNA break to a blunt end. Once the blunt end is made, the TdT will start adding dUTP in the terminal region of the DNA. dUTP is added until it forms a loop-like structure to make them close. What makes this assay particularly interesting is that TdT does not require any template strand to add nucleotide sequence to it, as it is a template-independent addition system. This means that dUTP will be incorporated depending on the amount of apoptosis going on inside the cell, as cell apoptosis leads to the breakdown of DNA. Hence, we can correlate apoptosis with the amount of TdT acting inside the cell. The dUTP are tagged with either enzymes or fluorescent tags. These modified dUTP when added will give a coloration when the assay will be running, giving an idea about cell apoptosis or cell death. This is because the dUTP will be added only to the broken DNA that are found in apoptotic cells.

Statistical analysis: All experiments included more than two independent repeats, and results were all represented with the standard deviation (SD). GraphPad PRISM 6 (GraphPad, San Diego, CA, USA) was employed for data analysis with one-way ANOVA or Student's t-test. All statistical significances were specified as p-values below 0.05.

Results

LUCAT1 was highly expressed in melanoma cells: It is reported that LUCAT1 is aberrantly upregulated in NSCLC, esophageal squamous cell carcinoma and clear cell renal cell carcinoma (9-11). We searched for the expression of LUCAT1 in melanoma cells. QRT-PCR assay found that LUCAT1 was highly expressed in melanoma cells (A2058, M21, A375 and A-875) compared with normal human keratinocytes (HaCaT) (see Figure 1A). Meanwhile, inhibition and over-expression efficiency of LUCAT1 were also searched in A2058 cells via qRT-PCR assay (see Figures 1B and 1C). In brief, LUCAT1 was highly expressed in melanoma cells.

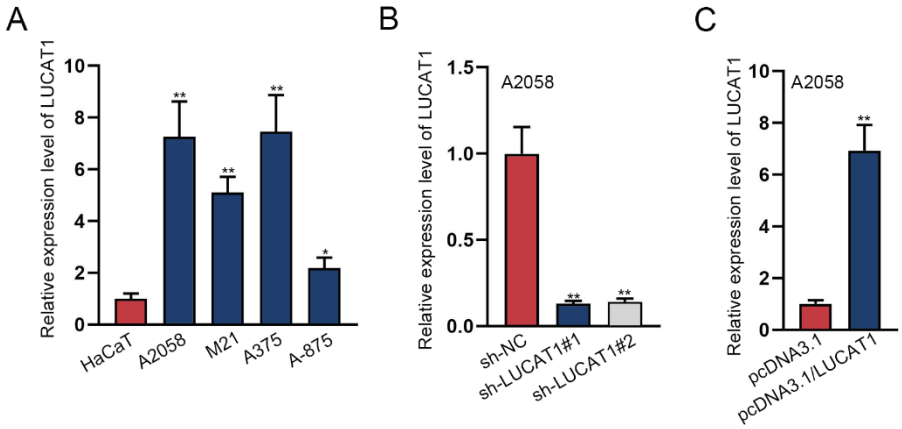

Figure 1: LUCAT1 was highly expressed in melanoma cells and their assay

A: Expression of lncRNA LUCAT1 was assessed via qRT-PCR assay in normal human keratinocytes (HaCaT) and melanoma cells (A2058, M21, A375 and A-875); B: Inhibition efficiency of lncRNA LUCAT1 in A2058 cells was tested via qRT-PCR assay; C: Over-expression efficiency of lncRNA LUCAT1 in A2058 cells was tested via qRT-PCR assay.

Over-expressed LUCAT1 can promote the progression of melanoma cells: The function of over-expressed LUCAT1 in melanoma cells was also identified. Colony formation and EdU assay found that the cell growth was further elevated by over-expressed LUCAT1 (see Figures 2A and 2B). Also, gain of function LUCAT1 also promoted cell migration and invasion via transwell assay (see Figure 2C). In short, over-expressed LUCAT1 can promote the progression of melanoma cells.

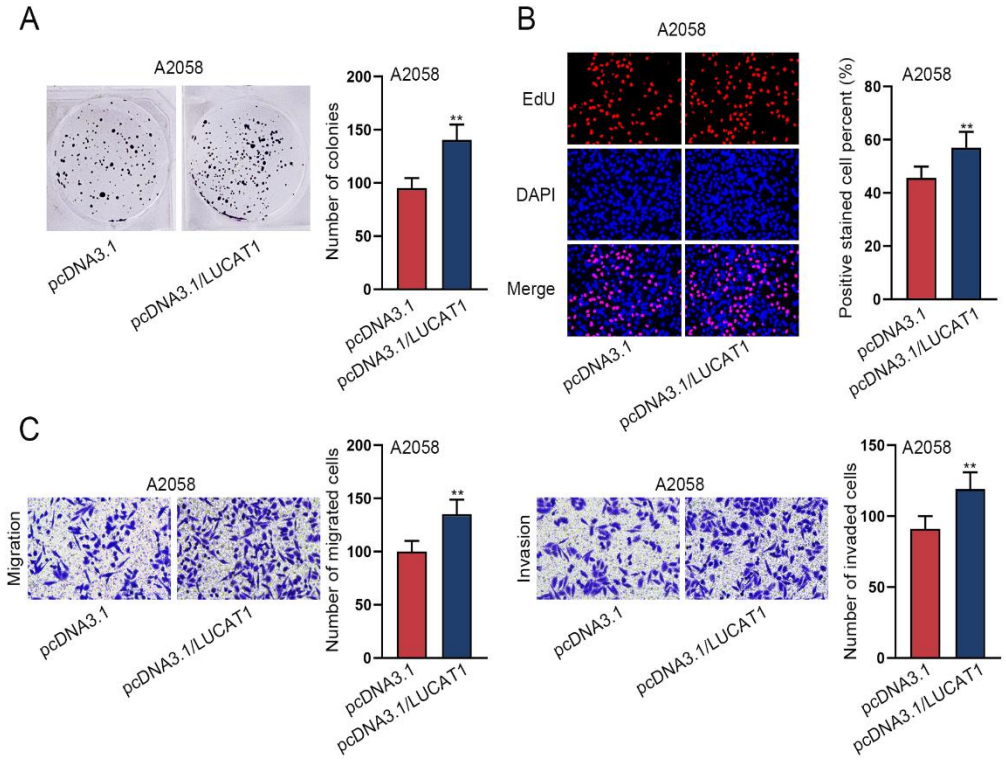

Figure 2: **Over-expressed LUCAT1 and progression of melanoma cells**

A and B: Colony formation assay and EdU assay investigated the cell proliferation of A2058 cells when LUCAT1 was over-expressed; C. Transwell assay searched the cell migration and invasion when LUCAT1 was over-expressed in A2058 cells.

LUCAT1 depletion could promote cell apoptosis in melanoma: Furthermore, we identified the regulation of LUCAT1 on melanoma cell apoptosis. According to the results of flow cytometry analysis and TUNEL, we found that cell apoptosis was significantly elevated via silenced LUCAT1 (see Figure 3B). Accordingly, LUCAT1 depletion can promote cell apoptosis in melanoma.

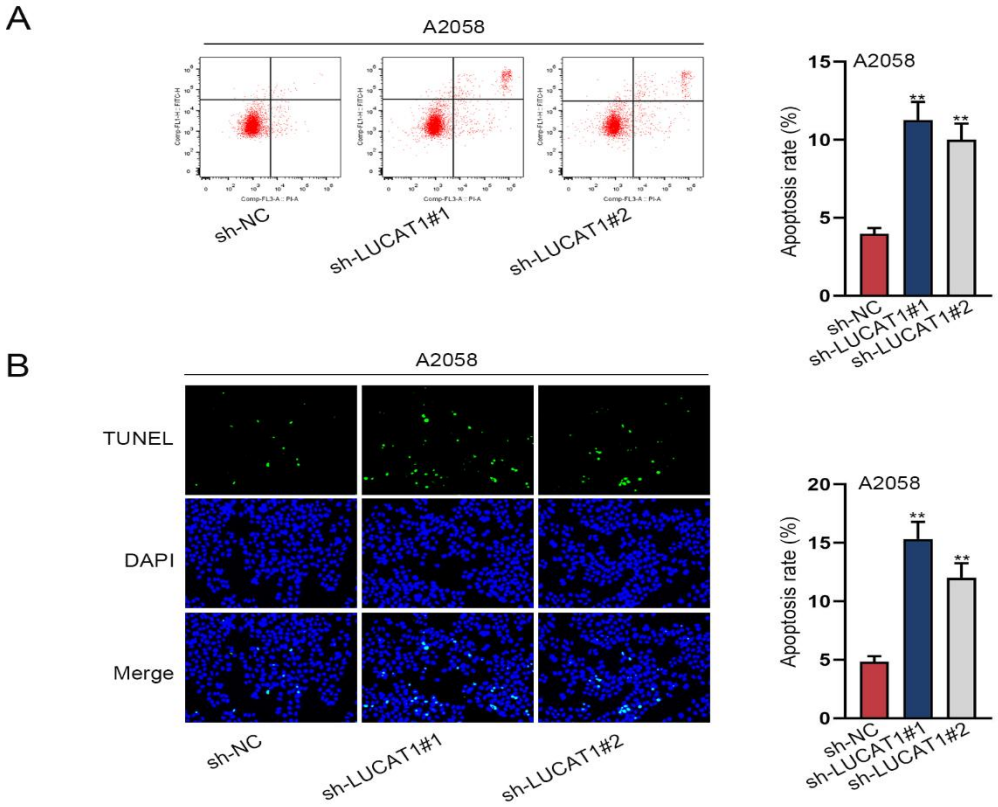

Figure 3: **LUCAT1 depletion and apoptosis in melanoma**

Silenced LUCAT1 can reduce the cell growth, migration and invasion of melanoma: We further identified the function of LUCAT1 in melanoma cells. Functional assays such as colony formation assay, EdU assay and transwell assay were conducted to search the cell proliferation, migration and invasion of A2058 cells. Colony formation assay and EdU assay found that cell growth was significantly inhibited by LUCAT1 depletion, as the number of colonies and positive stained cells were both decreased by LUCAT1 depletion (see Figures 4A and 4B). Transwell assay detected that cell migration and invasion were reduced by LUCAT1 depletion (see Figure 4C). In brief, silenced LUCAT1 can reduce the cell growth, migration and invasion of melanoma.

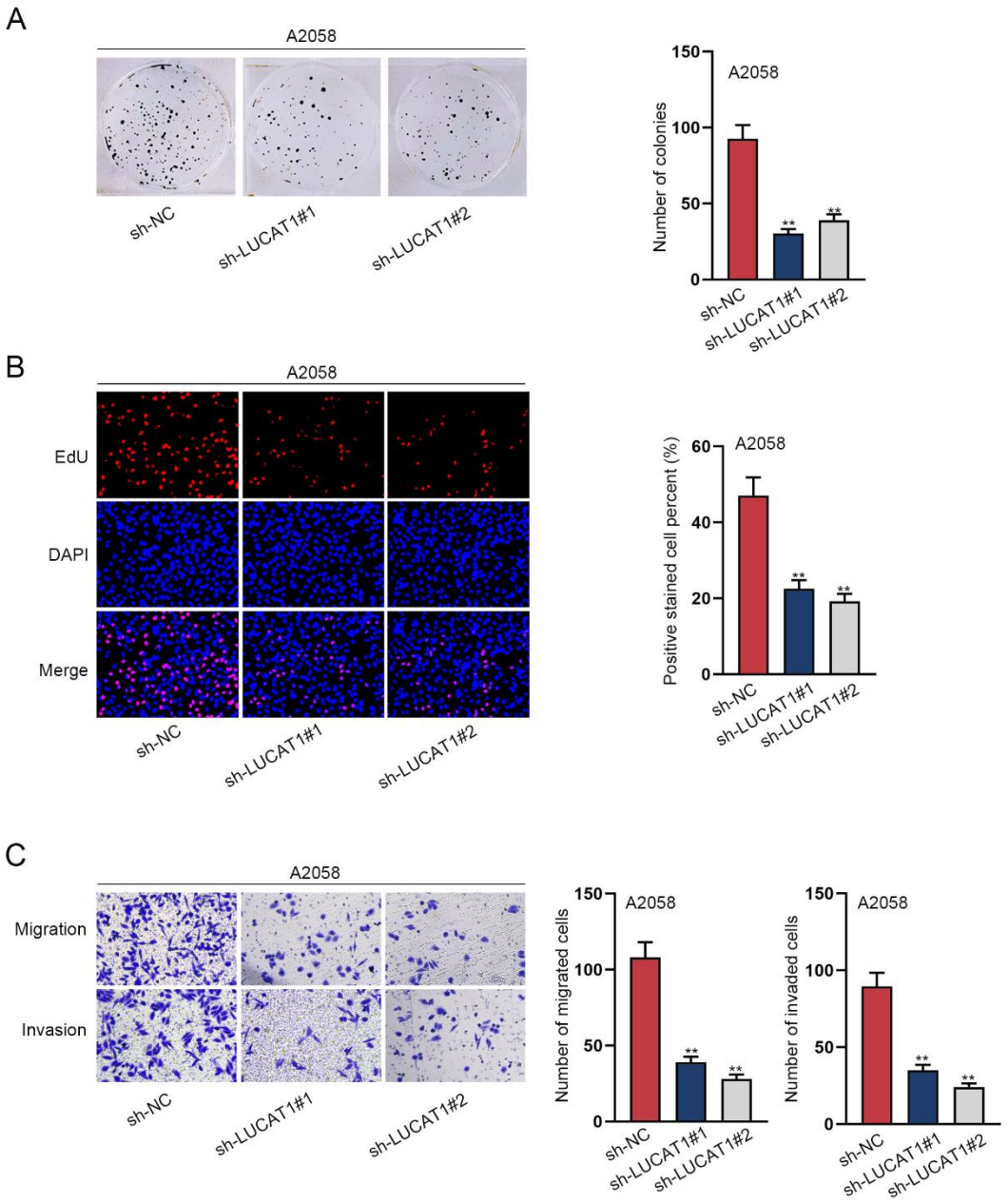

Figure 4: Silenced LUCAT1 and cell growth, migration and invasion of melanoma

A and B: Cell proliferation of A2058 cells was tested via colony formation assay and EdU assay when LUCAT1 was inhibited; C: Cell migration and invasion were identified via transwell assay when LUCAT1 was inhibited in A2058 cells.

Discussion

In our study, we detected the expression of lncRNA LUCAT1 in melanoma cells using qRT-PCR assay. We found that LUCAT1 was significantly upregulated in melanoma cells. Then, we further searched the role of LUCAT1 in melanoma. Previous studies have investigated the role of LUCAT1, finding that it can promote the progression of many cancers. For instance, it can activate cell growth of NSCLC and aggravate its progression by reducing the expression of p21 and p57 (11). Also, LUCAT1 can inhibit esophageal squamous cell carcinoma cell apoptosis by regulating the expression of DNMT1 (10). In addition, LUCAT1 can enhance the cell invasion and growth of clear cell renal cell carcinoma (9).

Colony formation assay and EdU assay found cell proliferation was significantly decreased by silenced LUCAT1 and activated by over-expressed LUCAT1. And cell migration and invasion were found inhibited

via LUCAT1 depletion and elevated by over-expressed LUCAT1 through transwell assay. Meanwhile, flow cytometry analysis and TUNEL assay found cell apoptosis was upregulated by silenced LUCAT1.

In the present study, we firstly identified the oncogene role of LUCAT1 in melanoma cells for providing more possible mechanism study in melanoma. The major findings of our study indicate that LUCAT1 is a potential therapeutic target and molecular biomarker for melanoma. LUCAT1 can address through the AKT/GSK-3β signaling pathway melanoma cell proliferation and invasion. Another necessary factor is that chemokine CXCL2 (macrophage inflammatory protein-2) is also responsible for the over-expression of LUCAT1 (9-11).

Conclusions

The study has attempted to portray the role of LUCAT1 in the development of melanoma. This work will further strengthen the research done in the field of cancer, offer a deeper insight into the causative and genetic mutations causing cancer, and will also enhance the advancement of melanoma research by providing information regarding the genes responsible for causing melanoma.

Acknowledgments

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