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Original Research Article

Regulatory mechanism of lncRNA miR143HG in miR-504 and its effect on proliferation and apoptosis of non-small cell lung cancer cells

Zhe Li*, Jinhua Liu, Yingqun Zhu, Qian Cai
Department of Pulmonary and Critical Care Medicine, The Third Hospital of Changsha, Changsha, Hunan 410015, China

For correspondence: **Email: yumiyin0529@163.com; **Tel:** +86-073189668355*

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Abstract

Purpose: *To investigate the expression and functional role of miR143HG in non-small cell lung cancer (NSCLC), and its effect on human lung adenocarcinoma cell behavior.*
Methods: *Differential expression of miR143HG between NSCLC tissues and healthy counterparts was identified through bioinformatic analysis. Subsequently, this expression difference in A549 and BEAS-2B cells was validated using quantitative polymerase chain reaction (qPCR). Overexpression of miR143HG in A549 cells was achieved through liposome-mediated transfection, while cell proliferation as well as apoptosis levels were assessed using cell counting kit-8 (CCK-8) assay, flow cytometry, and protein blotting. Expression of miR-504 (predicted as a target of miR143HG) was determined. Furthermore, A549 cells that overexpress both miR143HG and miR-504 were generated for comparison with cells overexpressing only miR143HG.*
Results: *Compared to BEAS-2B cells, A549 cells showed significantly reduced miR143HG and elevated miR-504 levels ($p < 0.05$). In A549 cells with miR143HG overexpression, cell proliferation and miR-504 expression significantly reduced while pro-apoptotic proteins (BAX, p53) significantly increased ($p < 0.05$). Simultaneous overexpression of miR143HG and miR-504 in A549 cells counteracted the effect of miR143HG alone, thus enhancing proliferation and diminishing pro-apoptotic protein expression, similar to control.*
Conclusion: *Downregulation of MiR143HG occurs in human lung adenocarcinoma cells. Upregulation of MiR143HG impedes A549 cell proliferation, promotes apoptosis through pro-apoptotic protein modulation and suppresses miR-504. Co-overexpression of miR143HG and miR-504 reverses these effects, resembling control conditions. Thus, miR143HG exerts its anti-cancer effect in A549 cells by modulating miR-504 and activating p53 pathway.*

Keywords: *lncRNA miR143HG; Non-small cell lung cancer; Proliferation; Apoptosis*

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INTRODUCTION

Long non-coding RNAs (lncRNAs) are a subclass of non-coding RNAs that have a length of more than 200 nucleotides, and either do not or only weakly code for proteins. LncRNA regulates gene performance and functionality during transcription, translation and post-translation [1]. Their ability to compete as endogenous RNAs with miRNAs to control gene expression is a key mechanism for their function [2]. Numerous studies have demonstrated that

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lncRNAs are important for tumor development and metastasis, and may act as biomarkers and indicators for diagnosis and treatment of cancer [3,4].

Non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer in China and has a high fatality rate [5]. Numerous poorly expressed lncRNAs have been identified in studies of non-small cell lung cancer (NSCLC), including oncogenic lncRNAs such as HOTAIR, MALAT1, and PVT1. These lncRNAs are up-regulated in lung cancer cells and promote the growth, invasion, and development of NSCLC cells. Additionally, there are oncostatic lncRNAs like MEG3, GAS5, and SPRY4-IT1 that are down-regulated in NSCLC and inhibit its progression [6]. The lncRNA, miR143HG, is located on chromosome 5, and is down-regulated in several malignant neoplasms including bladder cancer [7], hepatic carcinoma [8], squamous cell cancer of the larynx [9] and glioblastoma [10], in order to exert an oncogenic effect. The function and role of miR143HG in NSCLC have not been reported in the literature.

This current study was aimed at investigating the expression, and effect of miR143HG on the apoptosis and proliferation of non-small cell lung cancer cells (NSCLC), with the goal of strengthening diagnostic and therapeutic techniques. Furthermore, this study also investigated the underlying downstream signaling pathways by up-regulating the contents of target miRNAs in human lung adenocarcinoma cells.

EXPERIMENTAL

Cell culture and transfection

The human bronchial epithelial cell line (BEAS-2B) and NSCLC cell line (A549) (Shanghai Zhongqiao Xinzhou Biotechnology Co. Ltd. Shanghai, China) were maintained on Dulbecco's modified eagle medium (DMEM; Procell, Wuhan, China) with 10 % fetal bovine serum (FBS; Procell, Wuhan, China). The plates were incubated at 37 °C in an incubator with 5 % CO_2. Trypsin digestion and passaging were performed 24 h before transfection and cell density was allowed to reach 70 – 80 %. LncRNA miR143HG overexpression plasmid and its negative control plasmid were transfected into A549 cells following the Lipofectamine 2000 reagent instructions (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed in A549 cells 24 or 48 h after transfection to determine transfection efficiency.

Quantitative RT-PCR

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), reverse transcribed and amplified by RT-qPCR. Relative expression of miR-143HG and miR-504 were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

Cells from each treatment group were digested to obtain a cell concentration of 1 × 10^5 cells/mL, which was subsequently dispensed onto 96-well plates with 100 µL of cell solution in each well. Following attachment of the cells to the plate surface, 10 µL of CCK-8 solution (Jiangsu KeyGEN Biotech, Nanjing, China) was added at 0, 24, 48, 72 and 96 h respectively, and incubated for 4 h. Optical density at wavelength of 450 nm (OD450) was measured using an enzyme marker to generate the growth curve.

Flow cytometry

The cells were collected by centrifugation, resuspended with binding buffer, and subsequently stained with Annexin V- and propidium iodide (PI), following the instructions provided in the Annexin V-FITC Apoptosis Detection Kit. The cells were then incubated for 15 min at room temperature and shielded from light before being measured on a flow cytometer.

Western blot

Total cellular proteins were isolated from each batch of cells using a radioimmunoprecipitation assay (RIPA) lysate to determine protein content. Protein concentration was quantified with a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China).

Table 1: The primer sequence

Target	Forward primer	Reverse primer
miR143HG	5'-CAAGCCTCCCAACAGAAGAC-3'	5'-TACTCTGGATCCCCAGTCAG-3'
miR-504	5'-ACACTCCAGCTGGGGAGACC CTGGTCTGCACTC-3'	5'-CTCAACTGGTGTCGTGGA-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GGGAAACTGTGGCGTGAT-3'	5'-GAGTGGGTGTCGCTGTTGA-3'

Subsequently, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5 % skim milk for 2 h at room temperature. Specific antibodies against BAX (Abcam, USA), Bcl2 (Abcam, Cambridge, MA, USA), and p53 (Abcam, Cambridge, MA, USA) were incubated overnight at 4 °C at dilutions of 1:1000, 1:10, 000, and 1:100 respectively. After washing the membrane with Tris-buffered saline containing Tween-20 (TBST), the secondary antibody was added and incubated for 1 h at chamber temperature before exposure to assess the gray values of protein bands.

Statistical analysis

Statistical analysis was performed using Statistic Package for Social Science (SPSS) 26.0 (IBM, Armonk, NY, USA). Results from the two groups were compared using paired samples t-test or independent sample t-test, and $p < 0.05$ was considered statistically significant.

RESULTS

Expression of lncRNA miR143HG in NSCLC

Differential expression analysis was performed using lung cancer and lung cancer-free tissue samples from the Tumor Genome Atlas (TCGA) database. The size of the normal sample was 338 while the lung squamous carcinoma (LUSC) tumor sample size was 486. MiR143HG was found to be significantly under-expressed in study group (human lung adenocarcinoma cells) compared to control group (normal lung epithelial cells) ($p < 0.05$) (Figure 1 A). The qPCR results revealed that the expression of lncRNA miR143HG was significantly higher in study group compared to control group ($p < 0.05$) (Figure 1 B). Using the liposome transient method, A549 cells with increased miR143HG expression were generated and qPCR was carried out. miR143HG expression was significantly higher in the miR143HG overexpressed group compared to the negative control (NC) group ($p < 0.05$) (Figure 1 C), showing the success of developing A549 cell line that overexpresses miR143HG.

Effect of miR143HG overexpression on proliferation and apoptosis of A549 cells

The A549 cells overexpressing miR143HG were employed to examine the impact of miR143HG on growth and apoptosis of A549 cells. The CCK-8 revealed that the miR143HG group exhibited significantly lower proliferative activity compared to the NC group in A549 cells ($p < 0.05$) (Figure 2 A and B).

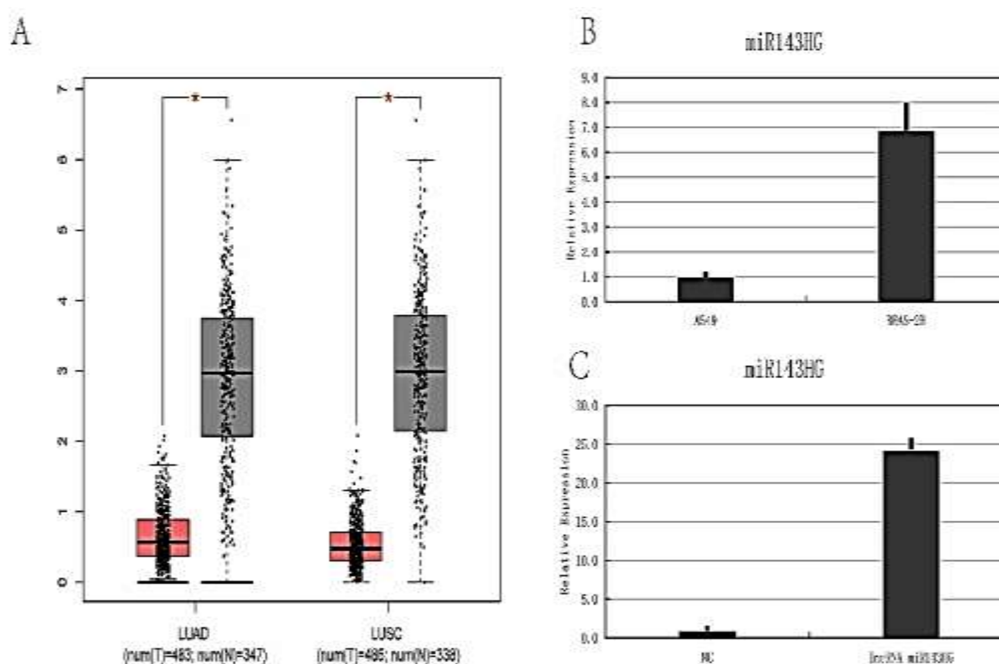

Figure 1: Expression of lncRNA miR143HG in NSCLC cells. (A) Analysis of the miR143HG gene differential expression in lung cancer and healthy lung tissue samples from the TCGA database; (B) Expression of lncRNA miR143HG in human lung adenocarcinoma cells and human normal lung epithelial cells detected by qPCR. (C) Validation of miR143HG transfection efficiency *$P < 0.05$

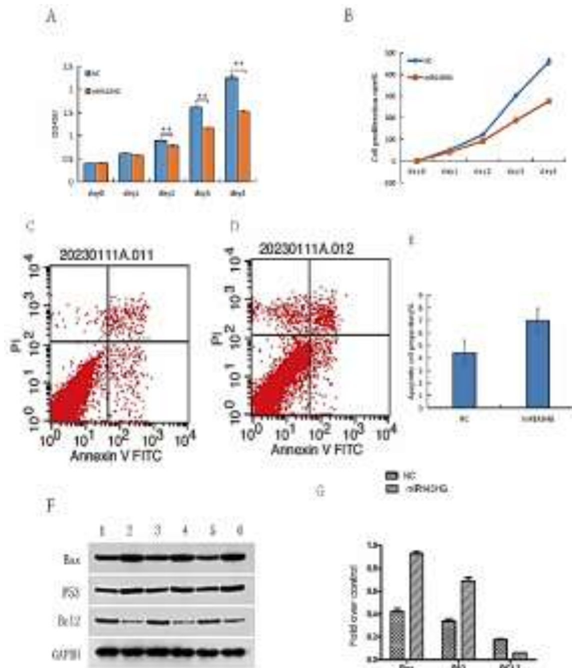

Figure 2: Effect of miR143HG overexpression on proliferation and apoptosis of A549 cells. (A-B) CCK-8 assay to detect cell proliferation in A549 cells in miR143HG high expression group versus negative control (NC) group. (C-E) Flow cytometry assessed apoptosis in both groups. (F-G) Western blotting to expression of the apoptosis-related proteins Bax, p53, and Bcl2 (1, 3, 5: NC; 2, 4, 6: miR143HG). *$P < 0.05$, **$p < 0.01$

Flow cytometry revealed that A549 cell apoptosis was significantly increased in miR143HG group compared to NC group ($p < 0.05$) (Figure 2 C, D and E). Western blotting results revealed that the miR143HG group significantly expressed apoptosis-related proteins (BAX, Bcl2 and p53) compared to NC group ($p < 0.05$) (Figure 2 F and G). These findings suggest that miR143HG may prevent A549 cells from proliferating and increase mortality rate.

Expression of miR-504 in A549

A probable target miRNA of lncRNA miR143HG was predicted using the starBase online database (http://starbase.sysu.edu.cn/), and it was discovered that this lncRNA shares a binding site with miR-504 (Figure 3 A). The qPCR result showed that A549 cells had significantly higher levels of miR-504 expression than normal lung epithelial cells ($p < 0.05$, Figure 3 B). Expression of miR-504 in A549 cells overexpressing lncRNA miR143HG was significantly lower compared to NC group ($p < 0.05$, Figure 3 C). These results suggest that miR-504 and miR143HG expression are negatively correlated. Also, A549 cells overexpressing both miR143HG and miR-504 were constructed and the results revealed that lncRNA miR143HG + miR-504 significantly increased the expression of miR-504 compared to the NC and miR143HG high-expression group alone ($p < 0.05$) (Figure 3 D). This implies that the development of the A549 cell line, which overexpresses miR143HG and miR-504, was effective.

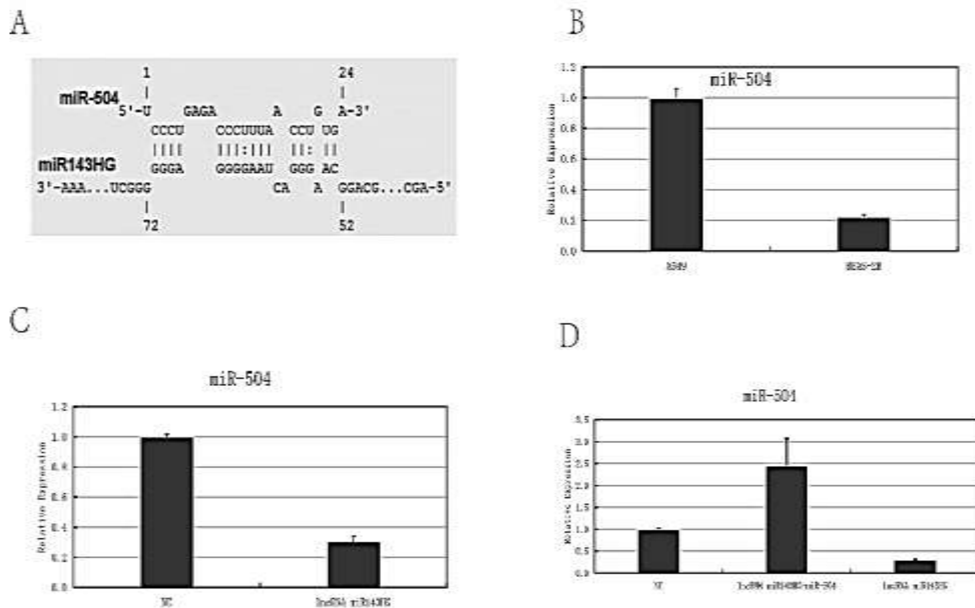

Figure 3: miR-504 expression in A549 cells. (A) miR143HG targeting association with miR-504; (B) qPCR assay for miR-504 expression in human lung adenocarcinoma cells and human normal lung epithelial cells; (C) qPCR detection of miR-504 expression in human lung adenocarcinoma transfected cells with high expression of miR143HG; (D) validation of miR-504 transfection efficiency

Effect of overexpression of miR-504 on proliferation and apoptosis in A549 cells

The miR-504 was involved in the proliferation and death of A549 cells by lncRNA miR143HG in A549 cells overexpressing both miR143HG and miR-504 and therefore, the downstream signaling pathway was examined. The proliferative activity of A549 cells was significantly enhanced in miR143HG + miR-504 group compared to the miR143HG-NC group ($p < 0.05$) (Figure 4 A, 4 B). The flow cytometry revealed that miR143HG + miR-504 group significantly decreased A549 cell apex when compared to miR143HG-NC group ($p < 0.05$) (Figure 4 C to F). Furthermore, miR143HG + miR-504 significantly decreased expression of apoptosis-related proteins (BAX, Bcl2, and p53) compared to miR143HG-NC group ($p < 0.05$) (Figure 4 G and H). These results suggest that the inhibitory and proapoptotic effects of miR143HG on A549 cells may be partially reversed by miR-504 overexpression.

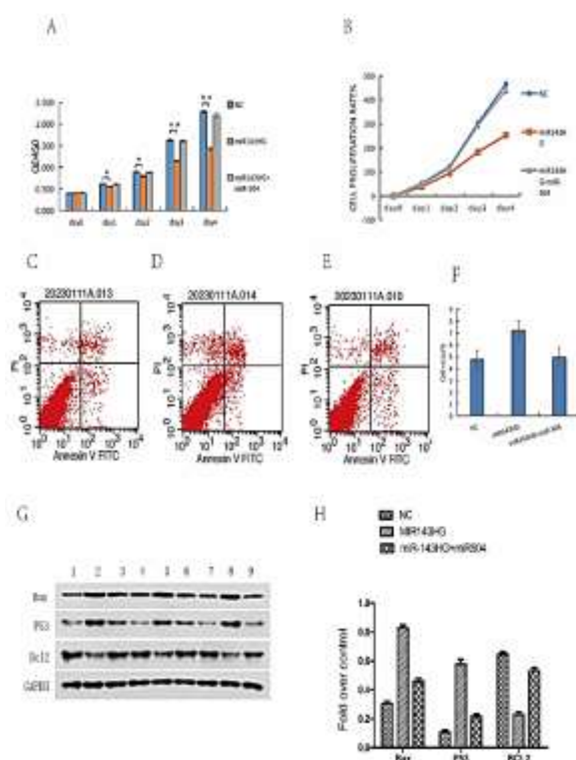

Figure 4: Effect of overexpression of miR-504 on proliferation and apoptosis of A549 cells. (A-B) CCK-8 assay to detect cell proliferation in A549 cells in miR143HG + miR-504 high expression group compared to miR143HG high expression and NC group; (C-F) Flow cytometry to detect apoptosis in all three groups; (G-H) Western blotting to determine the expression of apoptosis-associated proteins (Bax, p53 and Bcl2) (1, 4, 7: NC; 2, 5, 8: miR143HG; 3, 6, 9: miR143HG + miR-504) *$P < 0.05$, **$p < 0.01$

DISCUSSION

Lung cancers have the highest fatality rate of all malignant tumors [11]. They are grouped into two major groups including non-small cell lung cancer and small cell lung cancer based on the pathogenic and therapeutic characteristics. Non-small cell lung cancer makes up 85 % of all cases and is more prevalent than small cell lung cancer, as well as lung adenocarcinoma, lung squamous cell carcinoma, lung giant cell carcinoma and other histologic subtypes [12].

Early surgical resection is the main treatment and late targeted therapy combined with radiotherapy and immunotherapy is the current therapeutic option. Although progress has been made in molecularly targeted therapies, specific mechanisms of NSCLC pathogenesis cannot yet be fully elucidated given the diversity and complexity of pathogenic factors. lncRNAs are a diverse family of RNAs that do not have the ability to encode proteins but regulate genes in a variety of ways and play a role in various aspects of the growth of malignant tumors. Through induction, scaffolding, guidance, endogenous competing RNAs (ceRNAs) and other mechanisms, lncRNAs modulate cancer cell proliferation, polarization, invasion, metastasis and metabolic programming. They also exert oncogenic or inhibitory effects and play intricate and precise regulatory roles in cancer development [13].

Targeted non-coding RNA therapies have been used in tumor therapy with some success [14]. Therefore, it is fundamental to fully understand how lncRNAs interact with NSCLC. The ceRNA regulatory mechanism is the main mechanism by which lncRNAs function. lncRNA has multiple miRNA binding sites, which competitively adsorb and bind a large number of endogenous miRNAs, similar to a sponge, thus interfering with the expression of miRNA downstream, a regulatory mechanism known as ceRNA regulation [15]. The lncRNA miR143HG also has several miRNA binding sites, such as miR-143/145, miR-504, miR-21, miR-125a, miR-1275, etc. These sites allow the lncRNA to interact with a variety of target miRNAs to carry out several biological processes, including development of tumors, cardiovascular and airway diseases, endocrinological disorders and other diseases.

Xu *et al* [16] found that overexpression of lncRNA-miR143HG downregulates miR-143 expression, promotes ERK5 protein expression and phosphorylation and enhances ERK5-mediated cardiomyocyte protection. Zhang *et al* [17] showed that lncRNA miR143HG

competitively binds miR-1275 and mediates the ILK/Akt pathway involved in airway stenosis development. Xu *et al* [18] found that in order to regulate self-renewal and differentiation capabilities of bone marrow mesenchymal stem cells, which are engaged in bone production and bone resorption in estrogen-deficient osteoporosis, lncRNA miR143HG work in synergy with miR-143/145. In neoplastic diseases, lncRNA miR143HG acts mainly as an oncogene. When miR143HG is overexpressed, it interferes with the ability of miR-1275 to block the Wnt/-catenin signaling pathway, which prevents cancer cells from migrating, invading other tissues and proliferating [7]. In hepatocellular carcinoma, lncRNA miR143HG expression is also down-regulated and overexpression of miR143HG sponges miR-155 to inhibit Wnt/β-catenin signaling pathway and mitogen-activated protein kinase in order to suppress hepatocellular carcinoma cell growth and metastasis [8]. In endometrial cancer, lncRNA miR143HG upregulates p53 expression in cancer cells and promotes apoptosis by sponging miR-125a [19]. In glioblastoma, lncRNA miR143HG inhibits glioblastoma cell proliferation by sponging miR-504 to upregulate p53 expression [10]. In addition to sponging, lncRNA miR143HG acts by modulating epigenetic inheritance of target miRNAs. Xun *et al* [9] found that Overexpression of miR143HG boosted miR-21 methylation in laryngeal squamous cell carcinoma, which decreased cancer cell mobility and invasion rates.

In light of the significant role that lncRNA miR143HG plays in cancer, this study investigated the role and underlying motor mechanism of miR143HG in NSCLC. The findings demonstrated that human adenocarcinoma cells (A549) expressed significantly less miR143HG compared to healthy human lung epithelial cells (BEAS-2B). By liposome transient transfection, A549 cells overexpressing miR143HG were created and cell function experiments revealed that the proliferative activity of lung adenocarcinoma cells overexpressing miR143HG was significantly reduced. In lung adenocarcinoma cells overexpressing miR143HG, flow cytometry and Western blotting data showed a significant increase in apoptotic activity, which suggested that miR143HG suppressed multiplication and facilitated apoptosis in human lung adenocarcinoma cells. Further studies showed that miR-504 and miR143HG share a binding site and that the expression of miR143HG in human lung adenocarcinoma cells is negatively correlated. The proliferation of cells increased and apoptosis decreased in the lncRNA miR143HG + miR-504 group compared to A549 cells overexpressing lncRNA miR143HG alone comparable to the NC group, suggesting that overexpression of miR-504 partially counteract the inhibitory and pro-apoptotic effects of miR143HG.

CONCLUSION

The lncRNA miR143HG/miR-504/p53 pathway serves as a viable interventional target for the detection and therapeutic management of non-small cell lung cancer. This is because miR-504 may be targeted to activate the p53 signaling pathway, which in turn controls the ability of A549 cells to proliferate and undergo apoptosis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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