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A lncRNA Transcriptome Analysis of Phycocyanin-Treated Non-Small Cell Lung Cancer A549 Cell Lines

(Analisis Transkriptom lncRNA bagi Titisan Sel A549 Bukan Sel Kecil Kanser Paru-Paru yang Dirawat Fikosianin)

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ABSTRACT

Phycocyanin is a type of marine food additive with multiple biological properties, including anticancer activity, but its underlying antineoplastic mechanism in non-small cell lung cancer (NSCLC) remains unclear. To investigate the underlying regulatory mechanism of phycocyanin in NSCLC, a lncRNA microarray analysis was performed using a phycocyanin-treated A549 cell model. The classification and expression of lncRNAs were determined. The profiles of differentially expressed lncRNAs were generated and analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. The results showed that 193 lncRNAs were upregulated and 116 lncRNAs were downregulated in the phycocyanin-treated group compared with the control group, and qRT–PCR analysis confirmed the expression of selected lncRNAs. Bioinformatic analysis indicated that the differentially expressed lncRNAs and their target genes were enriched in the extracellular region, epithelium development, NOD-like receptor pathway, Notch signaling, and apoptosis process. In addition, coexpression network analysis identified 2,238 lncRNA–mRNA, lncRNA–lncRNA, and mRNA–mRNA pairs. In particular, 72 etc. differentially expressed lncRNA target genes were discovered in the interaction network, which provides insights into the potential mechanism of phycocyanin in A549 cells. Moreover, cell phenotype experiments showed that downregulating the expression of lncRNA ENST00000538717, a lncRNA that is downregulated after phycocyanin treatment, could significantly inhibit the migration and viability of A549 and H460 cells. In conclusion, this study lays a theoretical and potential foundation for NSCLC treatment and advances our understanding of the regulatory mechanisms of phycocyanin.

Keywords: A549 cells; anticancer; lncRNA; non-small cell lung cancer (NSCLC); phycocyanin

ABSTRAK

Fikosianin ialah sejenis bahan tambahan makanan laut dengan pelbagai sifat biologi, termasuk aktiviti antikanser, tetapi mekanisme antineoplastik asasnya dalam kanser paru-paru bukan-sel kecil (NSCLC) masih tidak jelas. Untuk mengkaji mekanisme pengawalseliaan asas fikosianin dalam NSCLC, analisis tatasusunan mikro lncRNA dilakukan menggunakan model sel A549 yang dirawat fikosianin. Pengelasan dan pengekspresan lncRNA telah ditentukan. Profil lncRNA yang dinyatakan secara berbeza dijana dan dianalisis menggunakan analisis *Kyoto Encyclopedia of Genes and Genom* (KEGG) dan *Gene Ontology* (GO). Keputusan menunjukkan bahawa 193 lncRNA telah dikawal dan 116 lncRNA telah dikurangkan dalam kumpulan yang dirawat fikosianin berbanding dengan kumpulan kawalan dan analisis qRT–PCR mengesahkan ekspresi lncRNA terpilih. Analisis bioinformatik menunjukkan bahawa lncRNA yang dinyatakan secara berbeza dan gen sasarannya diperkaya di kawasan ekstrasel, pembangunan epitelium, laluan reseptor seperti NOD, isyarat Notch dan proses apoptosis. Di samping itu, analisis rangkaian ekspresi bersama mengenal pasti 2,238 pasangan lncRNA–mRNA, lncRNA–lncRNA dan mRNA–mRNA. Khususnya, 72 dsb. gen sasaran lncRNA yang dinyatakan secara berbeza ditemui dalam rangkaian interaksi yang memberikan gambaran tentang mekanisme potensi fikosianin dalam sel A549. Selain itu, kajian fenotip sel menunjukkan bahawa perencatan pengawalaturan ekspresi lncRNA ENST00000538717, lncRNA yang mengalami perencatan pengawalaturan selepas rawatan fikosianin, boleh menghalang penghijrahan dan keviabelan sel A549 dan H460 dengan ketara. Kesimpulannya, kajian ini meletakkan asas teori dan potensi rawatan NSCLC dan meningkatkan pemahaman kita tentang mekanisme pengawalseliaan oleh fikosianin.

Kata kunci: Antikanser; fikosianin; lncRNA; kanser paru-paru bukan-sel kecil (NSCLC); sel A549

INTRODUCTION

Lung cancer remained the leading cause of cancer-related death in 2020, with an estimated 1.8 million deaths (Sung et al. 2021). Non-small cell lung cancer (NSCLC), a subtype of lung cancer, accounts for approximately 85% of lung cancer cases and is characterized by high mortality and a low cure rate (Ettinger et al. 2016; Molina et al. 2008). Currently, most NSCLCs are not effectively diagnosed at the early stage. Although treatments such as radiation therapy, chemotherapy or surgery have improved the survival rates of patients to a certain extent (Mansoori et al. 2017), the therapeutic efficacy of treatments for NSCLC is still very poor due to the lack of known biomarkers of NSCLC (Guo et al. 2019; Mansoori et al. 2017). Therefore, in-depth research to identify specific biomarkers of NSCLC is urgently needed for the development of new drugs that effectively treat NSCLC.

Long noncoding RNAs (lncRNAs) are a class of regulatory RNA molecules that are larger than 200 nucleotides and have no protein-coding ability (Ransohoff, Wei & Khavari 2018). Unlike microRNAs and small nucleolar/nuclear RNAs, lncRNAs have complex higher order structures (Novikova, Hennelly & Sanbonmatsu 2013). It has been shown that the ectopic expression of lncRNAs is relevant to the occurrence and metabolism of lung cancer. lncRNA TRERNA1 could promote the malignant progression of NSCLC by targeting FOXL1 (Luo et al. 2020). lncRNA SNHG14 also contributed to the growth of NSCLC via the miR-206/G6PD pathway (Zhao et al. 2020). In addition, some other lncRNAs, such as FENDRR, could exert antineoplastic effects on NSCLC cells by regulating the miR-761/TIMP2 axis (Zhang et al. 2019). Therefore, in-depth exploration of the regulatory role of lncRNAs in NSCLC and their mechanism is of great importance for the future treatment of NSCLC.

Phycocyanin, a pigment-protein complex derived from cyanobacteria, is a well-known natural food additive (Liu et al. 2016). It has been showed that phycocyanin, which is a potential natural antineoplastic factor, exerts antitumor effects on NSCLC (Li et al. 2015; Li et al. 2016). Previously, we performed a miRNA-seq analysis and discovered that miR-6883-3p, miR-3150a-3p, miR-642a-5p, and miR-627-5p were involved in the growth inhibitory effect of phycocyanin on NSCLC cells (Hao et al. 2022, 2021). However, few studies have investigated the lncRNA expression profiles in phycocyanin-treated NSCLC cell lines. As lncRNAs are the upstream regulators of miRNAs in cells (Bridges, Daulagala & Kourtidis 2021), further exploration of the expression profiles of lncRNAs participating in the phycocyanin-mediated antineoplastic process is vital to elucidating the mechanism of phycocyanin-mediated inhibitory effects on NSCLC.

In the present study, to further investigate the precise antineoplastic regulatory mechanism of phycocyanin in NSCLC, a lncRNA-seq was performed to establish the lncRNA profile of NSCLC A549 cells after phycocyanin exposure. The results of this work are expected to explore the anti-tumor mechanism of phycocyanin at the molecular level, which undoubtedly laid a theoretical basis for the potential treatment of NSCLC with phycocyanin and provided insights into the regulatory mechanism of phycocyanin.

MATERIALS AND METHODS

STUDY INFORMATION

This work included experimental techniques such as cell migration and cell viability assays, RNA extraction, RNA-seq assay, qRT–PCR, and bioinformatics analysis. The present work was performed in the labs of Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology and Business University in 2023.

CELL CULTURE AND SAMPLE TREATMENT

A549 and H460 cells were cultured in DMEM supplemented with 0.1 mg/mL streptomycin, 100 units/mL penicillin and 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO2 at 37 °C (American Type Cell Collection, ATCC, Manassas, VA, USA). Phycocyanin (95% purity) was purchased from Enviroloix (Portland, ME, USA). A549 cells were treated with phycocyanin or PBS as a control, followed by cell lysis and RNA extraction. The control groups were named Con-1, 2 and 3, and the phycocyanin-treated groups were named PC-1, 2 and 3.

RNA EXTRACTION AND QUALITY EVALUATION

The High Pure RNA isolation kit was employed for total RNA extraction according to the manufacturer's specification (Roche, Penzberg, Germany). A Qubit fluorometer (Life Technologies, Eugene, OR, USA) with the Qubit RNA Assay Kit and NanoDrop-1000 instrument were used to evaluate the quality of RNA (Thermo Fisher Scientific Inc., Waltham, USA). The Agilent Bioanalyzer microcapillary electrophoresis system was used to assess RNA quality (Agilent, Santa Clara, CA, USA).

RNA SEQUENCING

Two milligrams of polyA-minus RNA were used for cDNA library establishment. After total RNAs were extracted, mRNA and noncoding RNAs were enriched by removing rRNA, followed by fragmenting into short fragments (200-500 nt) using EpicentreRibo-Zero™ rRNA RemovalKit (Epicentre, WI, USA). RNA-sequencing libraries were generated under the instructions provided by NEB Next Ultra™ Directional RNA Library Prep kit for Illumina

(New England BioLabs, Inc.). The Illumina HiSeqTM 2000 Sequencing System by Berry Genomics Inc. (Beijing, China) was employed for sequencing. Differential expression analysis for lncRNA from the database has proceeded. The sequencing reads were mapped to the reference genome using the TopHat program. A coding potential calculator assay was employed for evaluation. The quality control of raw data was performed using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) (Morgenstern, Barzilay & Levin 2021; Nian et al. 2022; Taneja et al. 2023). TopHat2 (http://ccb.jhu.edu/software/tophat/index.shtml) (Corley et al. 2019; Kim et al. 2013; Rezansoff et al. 2019) was used for sequence comparative analysis after quality control. The Cufflinks software (http://cole-trapnell-lab.github.io/cufflinks/) was employed for transcript assembly.

ANALYSIS OF DIFFERENTIALLY EXPRESSED (DE) lncRNAs

The number of mapped tags that was given by the raw expression levels of that particular lncRNA. To correct for differences in tag count between samples, the tag counts were scaled to CPM (count per millions) based on the total number of aligned tags. The analysis of differentially expressed lncRNA between control and phycocyanin-treated samples was performed by edgeR. The setting was $|\log FC| > 1$ & FDR < 0.05.

TARGET GENES OF lncRNAs AND ENRICHMENT ANALYSIS

KEGG pathway analysis was employed for signal transduction pathway analysis of key genes. The *p*-value and gene count thresholds of KEGG analysis was *p*-adjust < 0.05 and 7, respectively. Gene Ontology (GO) analysis was used to identify important biological functions or processes of target genes. The *p*-value and gene count thresholds of GO analysis was *p*-adjust < 0.05 and 35, respectively.

qRT–PCR

qRT–PCR was performed by the Q6 qPCR system. GAPDH and β-actin were used as the endogenous control genes. SYBR Green Master Mix was used for signal detection. The 2-ΔΔCT method was used to calculate the expression of each gene. The primer sequences of lncRNA for qRT–PCR were as follows: forward: 5'-CGTTGCCTACAGTCAAGTCAGT-3';reverse: 5'-GCCATGGTTCCCTTACTGATAC-3'. The primer sequences of GAPDH and β-actin for qRT-PCR were as follows: Forward: 5'-TGTTCGTCATGGGTGTGAAC-3'; Reverse: 5'-ATGGCATGGACTGTGGTCAT-3' (GAPDH), Forward: 5'-GGAAATCGTGCGTGACATTAA-3'; Reverse: 5'-TCAGGCAGCTCGTAGCTCTT-3'.

CELL VIABILITY ASSAY

Cells were cultured in 96-well plates, followed by lncRNA inhibitor incubation for 24 h. The MTT method was employed to determine the viability of A549 cells at 24 and 48 h. Briefly, MTT was added to each well for 4 h, followed by HCl-SDS lysis buffer and the cells were incubated for 24 h. The absorbance at 570 nm was recorded using a microplate reader.

CELL MIGRATION ASSAY

Cells were incubated with lncRNA inhibitor and cultured in 6-well plates. A pipette tip was used to scratch the cells and create a 'wound'. Each wound was observed at three different positions and its width was recorded. The three width values were averaged. The widths of the wounds were observed at 0 and 24 h. The cell migration rate was calculated from the difference in scratch width at 24 h over the original width (0 h).

STATISTICAL ANALYSIS

The data were analyzed by Microsoft Excel and GraphPad Prism. A two-tailed Student's t test (paired t-test) was used for data analysis, and $p < 0.05$ indicated a significant difference between the control and test groups.

RESULTS AND DISCUSSION

CLASSIFICATION AND SUBGROUP ANALYSIS OF lncRNAs

Phycocyanin has been reported to exert effective antineoplastic effects on NSCLC cells (Li et al. 2016, 2015). Further investigation of the underlying anticancer mechanism of phycocyanin can help clarify the pathogenesis of NSCLC, laying the theoretical basis for lung cancer treatment. lncRNAs are well-known important molecules that regulate gene expression at the transcriptional, epigenetic, and posttranscriptional levels (Ren et al. 2017). Numerous lncRNAs have been identified by high-throughput technology and are related to growth and development in cancer cells.

Over 26,000 lncRNAs (including known and novel lncRNAs) were identified in the Con and PC groups (Table S1). The chromosomal distribution and transcript types of lncRNAs were determined. In Table S2, of the antisense lncRNAs, the known lncRNAs accounted for 93.5%. Of the sense intron overlap, the known lncRNAs accounted for 96.1%. Of the intergenic lncRNAs, the known lncRNAs accounted for 99.4%. Of the sense exon overlap, the known lncRNAs accounted for 95.0%. Of the bidirectional lncRNAs, the known lncRNAs accounted for 99.9%.

ANALYSIS OF lncRNA EXPRESSION AND DIFFERENTIALLY EXPRESSED (DE) lncRNAs

As shown in Figure 1(A), the lncRNA expression patterns of the Con and PC samples were similar, indicating the suitable replication of each experiment. Finally, 5,084 and 6,080 lncRNA transcripts were obtained from the Con and PC groups, respectively, after eliminating the single-exon, low-expression, and unreliable fragments. Of these, 4886 lncRNAs were found in both Con and PC samples (Figure 1(B)). Table S3 presents the top 15 lncRNAs that are expressed in the Con and PC groups based on TPM (transcripts per million reads). Strikingly, some lncRNAs were highly expressed in both the Con and PC groups, including ENST00000602361, ENST00000554988, ENST00000618589, and ENST00000659240. Some lncRNAs exhibited sample-specific expression. For instance, NONHSAT135828.2 and ENST00000508832 were specifically highly expressed in phycocyanin-treated A549 cells, while NONHSAT190639.1 and NONHSAT176665.1 were specifically highly expressed in control cells.

The heatmap of differentially expressed lncRNAs showed that the expression patterns of lncRNAs within Con or PC samples were consistent, while the expression profiles between Con and PC groups exerted extremely significant differences (Figure 1(C)), indicating the effects of phycocyanin on the expression of lncRNAs in A549 cells. In total, 309 lncRNAs exhibited significant expression differences between the Con and PC groups, including 193 significantly upregulated and 116 significantly downregulated lncRNAs after phycocyanin treatment in A549 cells (Figure 1(D)). The scatter diagram of DE lncRNAs is shown in Figure 1(E). The detailed information of DE lncRNAs were shown in Table S4. These lncRNAs are likely to be key lncRNAs that may act as downstream targets of phycocyanin in NSCLC cells. Table S5 shows the top 10 downregulated and top 10 upregulated lncRNA transcripts.

GO AND KEGG ENRICHMENT ANALYSES OF DE LNCRNAS AND PREDICTED TARGETS

The DE lncRNAs were annotated by KEGG and GO enrichment analyses. Figure 2(A) shows the top 20 GO entries. Moreover, several of the GO items were related to cell activities, including GO:0006955 (immune response), GO:0070613 (regulation of protein processing), GO:2000257 (regulation of protein activation cascade), GO:1903034 (regulation of response to wounding), and GO:0006952 (defense response). Figure 2(B) shows the KEGG pathway enrichment results. The DE lncRNAs were shown to be enriched in signaling pathways related to cell growth and metabolism, such as the NOD-like receptor signaling pathway (map04621) and apoptosis (map04215). Strikingly, the DE lncRNAs were enriched in pathways associated with cancer (map05200) and small cell lung cancer (map05222) according to the KEGG analysis, suggesting that the DE lncRNAs affected the growth, apoptosis, and viability of A549 lung cancer cells, which were possibly regulated by phycocyanin.

The potential target genes of DE lncRNAs were predicted by a trans-regulation analysis method to determine the functions of these core lncRNAs. A total of 207 trans targets (p-adjust < 0.01) for DE lncRNAs were screened and obtained. Detailed information on the potential targets of lncRNAs is shown in Table S6. Next, the target genes were annotated by GO enrichment analysis. In total, 780 GO entries were obtained, and Figure 2(C) presents the top 20 GO entries. It is worth noting that the DE lncRNAs were enriched in several GO items related to cell movement and proliferation were enriched, including GO:0007166 (cell surface receptor signaling pathway), GO:0006928 (movement of cell or subcellular component), GO:0006955 (immune response), and GO:0070613 (regulation of protein processing). Moreover, the Notch signaling pathway (GO:0007219) was predicted to be activated in A549 cells after phycocyanin treatment.

In this work, 193 upregulated and 116 downregulated lncRNAs were identified between phycocyanin-treated and control A549 cells. In addition, the target genes (mRNAs) of DE lncRNAs were identified in PC samples, indicating that the expression of various genes was altered by lncRNAs after phycocyanin treatment. Currently, it has been reported that lncRNAs could be potential biomarkers for multiple cancers, including NSCLC (Khandelwal et al. 2015; Zhen et al. 2018); however, the involved regulatory mechanism is not yet clearly understood. In this work, according to the GO enrichment analysis, the biological factors affected most by phycocyanin are extracellular region, extracellular space, and epithelium development, which suggested that NSCLC cell behavior is likely regulated by phycocyanin through lncRNAs and target genes. In addition, KEGG analysis of lncRNAs showed that the NOD-like receptor pathway, Notch pathway, and apoptosis were also enriched after phycocyanin exposure in A549 cells. Alzokaky et al. (2020) reported that phycocyanin could protect against ethanol-induced gastric ulcers in rats through multiple signaling pathways, including the HMGB1/NLRP3/NF-κB and NOD-like receptor pathways. It is worth mentioning that we used mRNA-seq to study the potential mechanism of phycocyanin, which also showed that 'apoptosis pathway' and 'NOD-like receptor pathway' were significantly enriched after phycocyanin explosure (Hao et al. 2018). The similar conclusions of the two studies have reflect the potential regulatory mechanism of phycocyanin on NSCLC cells.

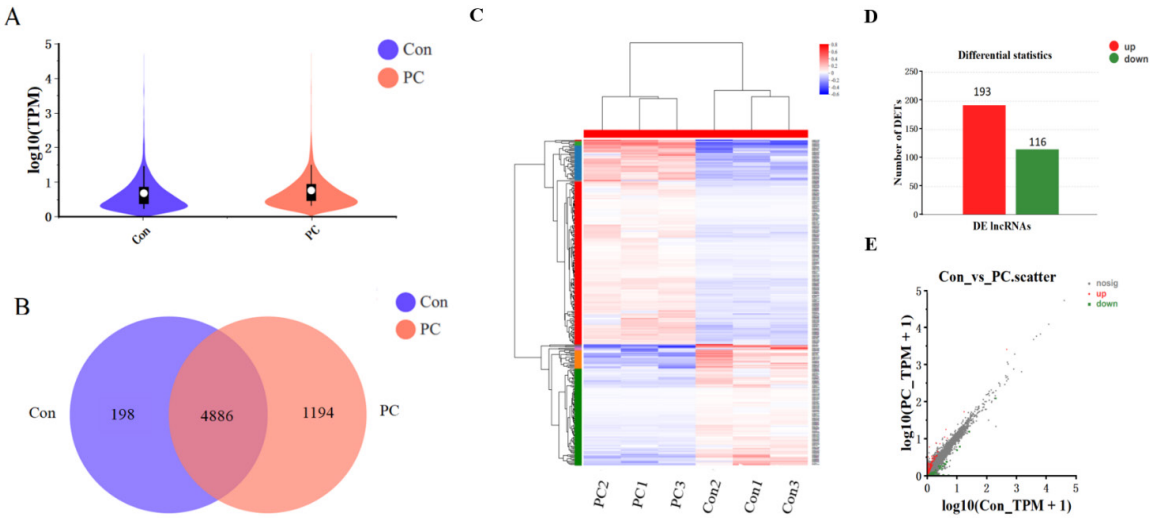

FIGURE 1. The expression analysis of lncRNAs. (A) The violin figure of expression pattern of lncRNAs in Con and PC groups, (B) Venn figure of the lncRNA numbers in Con and PC groups, (C) Heatmap of DE lncRNAs in each sample. Blue indicates low expression levels, red indicates high expression levels, (D) Number of DE lncRNAs between Con and PC groups, and (E) Scatter diagram of DE lncRNAs. Red indicates upregulated lncRNAs, and green represents downregulated lncRNAs

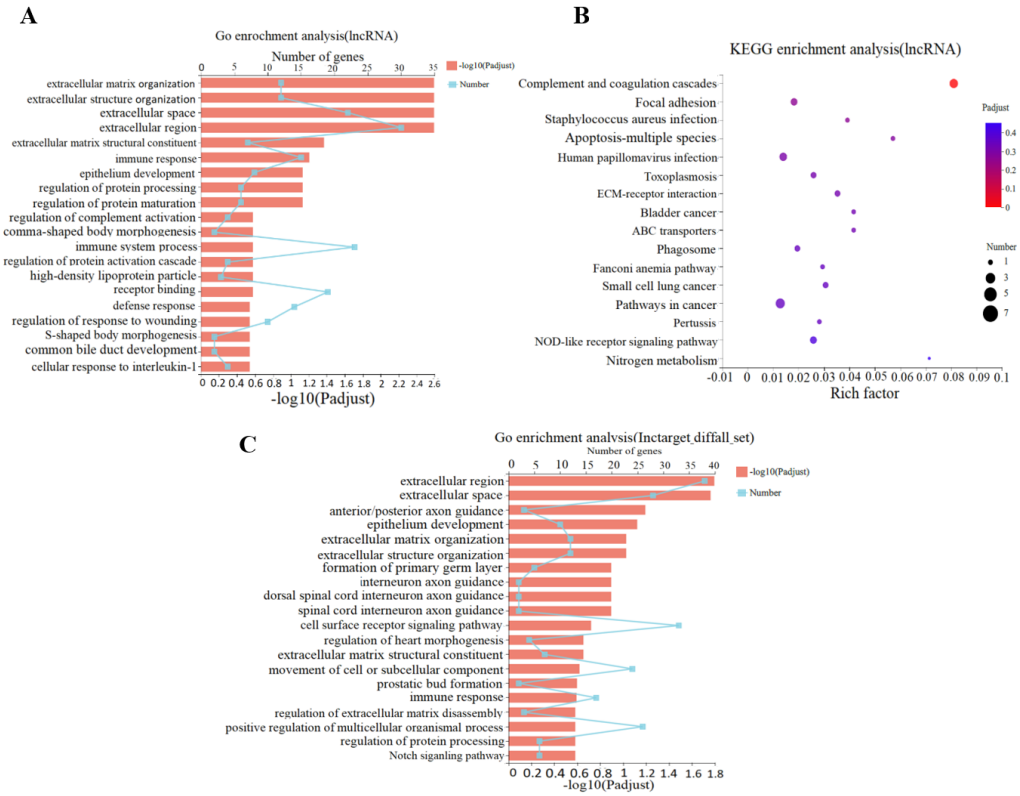

FIGURE 2. The GO (A) and KEGG, (B) enrichment analysis of DE lncRNAs, and (C) The GO enrichment analysis of targets of DE lncRNAs

ESTABLISHMENT OF THE lncRNA–mRNA AND TARGET GENE COEXPRESSION NETWORKS

As shown in Supplementary Figure S1, a comprehensive lncRNA–mRNA coexpression network was constructed. A total of 2,238 mRNA–mRNA, lncRNA–lncRNA, and lncRNA–mRNA pairs were identified, showing the potential mutual effects of DE lncRNAs and mRNAs. Detailed information on lncRNAs and mRNAs is presented in Table S7. It is worth noting that ENSG00000130600 (lncRNA) and ENSG00000172638 (mRNA) showed the highest negative correlation coefficient (p-adjust < 0.01), while ENSG00000258479 (lncRNA) and ENSG00000188747 (mRNA) showed the highest positive correlation coefficient (p-adjust < 0.01). In addition, one mRNA with high correlation coefficient (such as ENSG00000188747) might have a regulatory relationship with multiple mRNAs and lncRNAs, including ENSG00000008517 (mRNA), ENSG00000078098 (mRNA), and ENSG00000254815 (lncRNA). The lncRNA–mRNA network showed the underlying complex regulatory mechanisms of phycocyanin in A549 cells, providing a potential theoretical basis for NSCLC treatment.

To further investigate the interactions between DE lncRNAs and target genes, an interaction network was constructed. As shown in Figure 3(A), 72 target genes were present in the interaction network. Several genes were found to exert high node degree in the network, including cadherin 1 (CDH1, node degree (Zhen et al. 2018), SRY-box transcription Factor 9 (SOX9, node degree G. (Zhang et al. 2019), and serpin family A member 1 (SERPINA1, node degree (Luo et al. 2020), which suggests that these genes probably play vital roles in phycocyanin-mediated regulatory processes via lncRNAs in A549 cells. Moreover, lncRNAs also regulated multiple target genes in a cross-talk manner (Figure 3(B)). lncRNA ENST00000662245 was downregulated in phycocyanin-treated A549 cells and regulated the expression of CDH1, SOX9, and VTN (vitronectin). Additionally, ENST00000538717 was downregulated in the PC groups and affected the expression of IGF2 and MBF. Coincidentally, the mRNA-seq in our previous work have discovered multiple pathways and signal molecules involved in phycocyanin-mediated inhibition of NSCLC cells. Interfering a series of signal pathways and some key node factors such as MMP and p53, phycocyanin influenced the growth of NSCLC cells. Interestingly, the MMP protein family both showed significantly differences in present work and our previous study (Hao et al. 2018), including MMP1 (Figure 3(C)) in present work) and MMP3, MMP9, MMP14 (in previous study), indicating lncRNA might suppress cell migration through MMP protein family, which supported the results of previous work. The former study just focused on study the mRNAs levels, which could be regulated by its upstream lncRNAs. So, the present work is a further extension and expansion of previous work. In a word, these lncRNA–mRNA interaction pairs may play a crucial regulatory role in A549 cells after phycocyanin exposure.

The discovery of this lncRNA–mRNA network deepened the understanding of the pathogenesis of NSCLC and the antineoplastic mechanism of phycocyanin. Zhang et al. (2021) showed a lncRNA–mRNA coexpression network for myocardial infarction, providing diagnosis and prognosis biomarkers of myocardial infarction by means of specific lncRNA expression profiles. Moreover, Cai et al. (2021) constructed a lncRNA–mRNA ceRNA network, discovering novel prognostic biomarkers in rectal cancer. These reports suggest that lncRNAs could be important regulators of mRNAs and gene expression in various diseases, including cancer. In particular, lncRNA SOX2-OT was found to reduce the efficacy of therapy and worsen the clinical prognosis of lung cancer patients through AKT/ERK and SOX2/GLI-1 signaling (Marcela Herrera-Solorio et al. 2021). It is worth noting that in the current study, the transcription factor SOX9, from the same family as SOX2, was shown to be regulated by two differentially expressed lncRNAs, ENST00000662245, ENST00000432735 and NONHSAT254537.1, showing the potential regulatory mechanism of phycocyanin in A549 cells. In addition, 2,238 mRNA–mRNA, lncRNA–lncRNA, and lncRNA–mRNA pairs were identified, showing the potential mutual effects of DE lncRNAs and mRNAs in this study. These molecules may be biologically important in the phycocyanin-mediated anticancer effects on NSCLC cells, which will be under investigation in the future.

VALIDATION AND FUNCTIONAL ASSAYS OF lncRNAs IN NSCLC CELLS

As shown in Figure 3(B), several targets were enriched in the functional category of cell proliferation and movement progress (GO: GO:0007219). Of these lncRNAs, lncRNA ENST00000538717 was predicted to downregulate (log2FC = -10.02) and regulate multiple genes (Figure 4(A)), including IGF2 (insulin-like growth Factor 2), DHRS2 (dehydrogenase/reductase 2), BMF (Bcl2 modifying factor), and RND1 (Rho family GTPase 1), indicating that ENST00000538717 may regulate the viability of phycocyanin-treated A549 cells. Next, to verify the RNA-seq results, qRT–PCR analysis was used to measure the expression of ENST00000538717 in a phycocyanin-treated NSCLC cell model. As shown in Figure 4(B), the relative expression of lncRNAs was significantly downregulated after phycocyanin exposure in both H460 and A549 cells, suggesting that the sequencing results are

highly reliable. To further estimate whether the ectopic expression of ENST00000538717 affects the growth and migration of NSCLC cells, cell survival and wound healing assays were employed. Figure 4(C) and 4(D) shows that suppressing the expression of ENST00000538717 could significantly decrease the viability and migration in both A549 and H460 cells. Altogether, the results showed that ENST00000538717 might be a candidate regulator in phycocyanin-mediated inhibition of NSCLC cell viability.

Increasing evidence has suggested that lncRNAs could be important prognostic factors for tumors, including lung cancer (Huang et al. 2020; Loewen et al. 2014; Zhen et al. 2018). Zhang et al. (2018) reported that lncRNA TUC338 was overexpressed in lung cancer and promoted the invasion of lung cancer through the MAPK pathway. In addition, lncRNAs could also act as cancer suppressor genes in NSCLC through lncRNA–lncRNA interactions (Wan et al. 2021). In the current work, a total of 309 lncRNAs, including 116 downregulated and 193 upregulated lncRNAs, exhibited significant expression differences between the Con and PC groups. lncRNA ENST00000538717 was chosen to verify the lncRNA-seq results, and its expression was downregulated after phycocyanin treatment, indicating that it is an inhibitory factor in NSCLC cells. Interestingly, ENST00000538717 performs different functions in different types of tumors. Yi, Li and Sun (2021) reported that overexpression of LINC00852 promotes prostate cancer cell proliferation and metastasis. However, our results showed that ENST00000538717 impaired the viability and migration of NSCLC cells, which was consistent with Tuo's study (Tuo, Liang & Zhou 2021). The data suggested that these lncRNAs may be novel regulatory factors of phycocyanin in NSCLC cells, and the precise functions of lncRNAs remain under further investigation.

In summary, the present work systematically identified the changes in lncRNAs during the process of phycocyanin-mediated inhibition of NSCLC cell activity and further characterized these changes through bioinformatic analyses. A key differential lncRNA was screened and verified, and phycocyanin was found to decrease the activity of NSCLC cells *in vitro* by inhibiting this lncRNA. Nowadays, competing endogenous RNAs (ceRNAs) hypothesis has become an important research field in the study of cell life activity and tumor regulation (Chen et al. 2021). Studies have shown that the lncRNA-mRNA regulatory network is involved in the regulation of various tumor cells (Chen et al. 2021; Guo et al. 2021; Wu et al. 2020). As a result, functional study of specific lncRNA in cancer cells has certain limitations. The combination of lncRNA-mRNA network with cell phenotype is the key research direction of ceRNAs. Meanwhile, beside lncRNA, ceRNAs including cirRNA, pseudogene, competing miRNA can also interfere with expression of target mRNAs (Qi et al. 2015). Therefore, further explorations are urgently needed to dig out the deep anti-tumor mechanism of phycocyanin, which is also the topic of our future research.

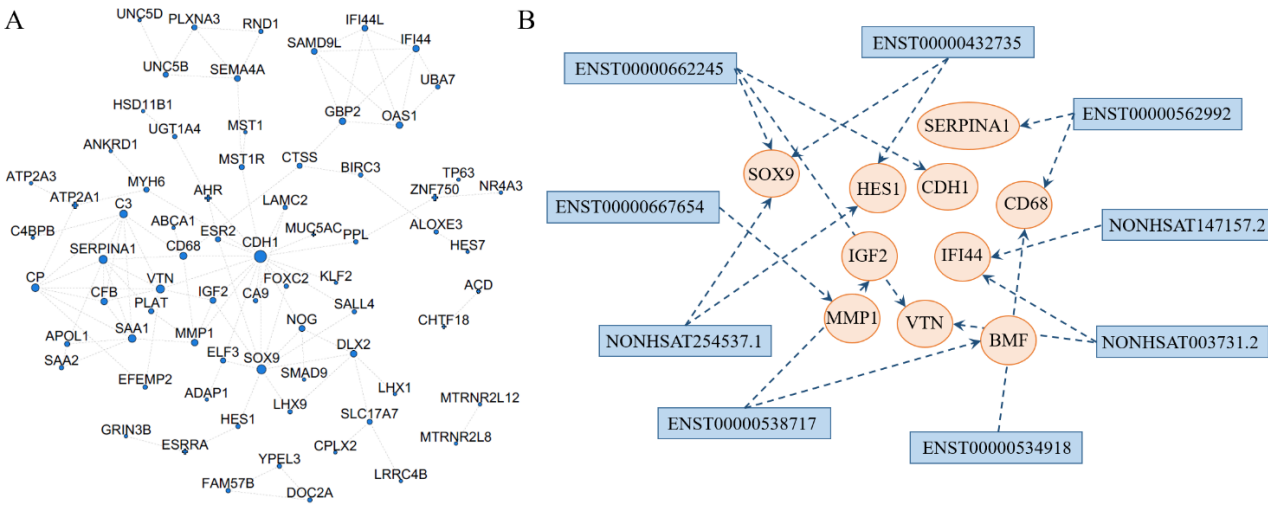

FIGURE 3. The lncRNA-mRNA, and target gene co-expression network. (A) Interaction network of DE lncRNA targets, and (B) The lncRNA-mRNA interaction pairs with high positive correlation coefficient

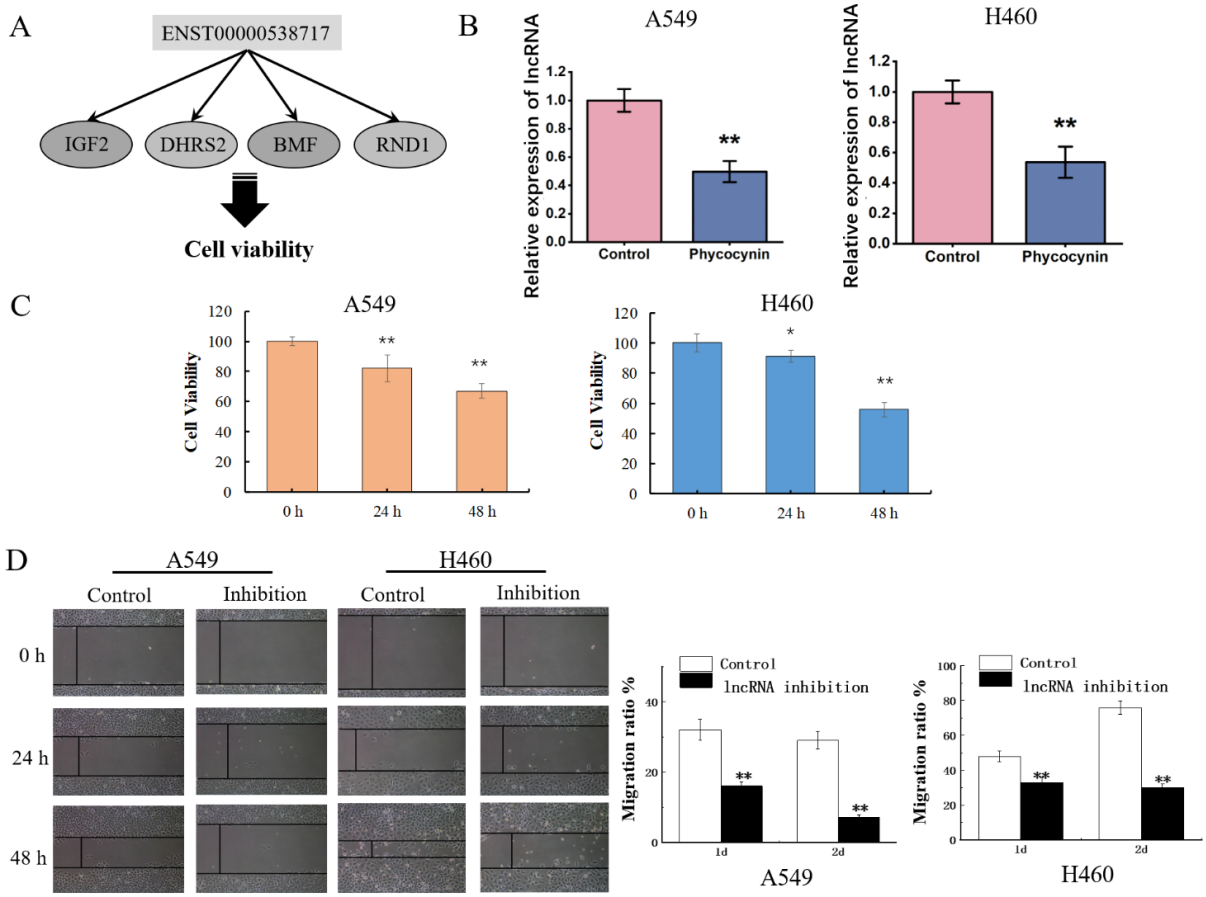

FIGURE 4. Validation and functional assays of lncRNA ENST00000538717 in NSCLC cells. (A) The target prediction and GO analysis indicate that lncRNA ENST00000538717 are related to multiple genes and cell viability, (B) qRT-PCR validation of RNA-sequencing expression result of lncRNA ENST00000538717 in A549 and H460 cells, (C) Cell survival analysis of A549 and H460 cells after ENST00000538717 inhibition, and (D) Cell migration analysis of A549 and H460 cells after ENST00000538717 inhibition. *, $p < 0.05$, **, $p < 0.01$

CONCLUSION

In this study, differentially expressed lncRNAs and target genes were discovered, and they are involved in cell viability, apoptosis, migration and corresponding signaling pathways. Moreover, the lncRNA–mRNA interaction network provided a novel regulatory pattern, which showed potential molecular targets of phycocyanin, molecular therapeutic factors and prognostic markers of NSCLC. Most notably, 72 significant gene nodes were discovered in the target gene interaction network, which are tightly associated with the antineoplastic mechanism of phycocyanin in NSCLC. The results may provide a new strategy for further investigation of antitumor targets and the regulatory process of phycocyanin in NSCLC.

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REFERENCES

Alzokaky, A.A.M., Abdelkader, E.M., El-Dessouki, A.M., Khaleel, S.A. & Raslan, N.A. 2020. C-phycocyanin protects against ethanol-induced gastric ulcers in rats: Role of HMGB1/NLRP3/NF-kappa B pathway. *Basic & Clinical Pharmacology & Toxicology* 127(4): 265-277. https://doi.org/10.1111/bcpt.13415

Bridges, M.C., Daulagala, A.C. & Kourtidis, A. 2021. LNCcation: lncRNA localization and function. *Journal of Cell Biology* 220(2): e202009045. https://doi.org/10.1083/jcb.202009045

Cai, G., Sun, M., Li, X. & Zhu, J. 2021. Construction and characterization of rectal cancer-related lncRNA-mRNA ceRNA network reveals prognostic biomarkers in rectal cancer. *Iet Systems Biology* 15(6): 192-204. https://doi.org/10.1049/syb2.12035

Chen, L., Zou, W., Zhang, L., Shi, H., Li, Z. & Ni, C. 2021. ceRNA network development and tumor-infiltrating immune cell analysis in hepatocellular carcinoma. *Medical Oncology* 38(7): 85. https://doi.org/10.1007/s12032-021-01534-6

Corley, S.M., Troy, N.M., Bosco, A. & Wilkins, M.R. 2019. QuantSeq. 3' sequencing combined with Salmon provides a fast, reliable approach for high throughput RNA expression analysis. *Sci. Rep.* 9(1): 18895. https://doi.org/10.1038/s41598-019-55434-x

Ettinger, D.S., Wood, D.E., Akerley, W., Bazhenova, L.A., Borghaei, H., Camidge, D.R., Cheney, R.T., Chirieac, L.R., D'Amico, T.A., Dilling, T.J., Dobelbower, M.C., Govindan, R., Hennon, M., Horn, L., Jahan, T.M., Komaki, R., Lackner, R.P., Lanuti, M., Lilenbaum, R., Lin, J., Loo Jr., B.W., Martins, R., Otterson, G.A., Patel, J.D., Pisters, K.M., Reckamp, K., Riely, G.J., Schild, S.E., Shapiro, T.A., Sharma, N., Stevenson, J., Swanson, S.J., Tauer, K., Yang, S.C., Gregory, K. & Hughes, M. 2016. NCCN Guidelines (R) Insights: Non-small cell lung cancer, Version 4.2016 featured updates to the NCCN guidelines. *Journal of the National Comprehensive Cancer Network* 14(3): 255-264. https://doi.org/10.6004/jnccn.2016.0031

Guo, K., Qian, K., Shi, Y., Sun, T. & Wang, Z. 2021. LncRNA-MIAT promotes thyroid cancer progression and function as ceRNA to target EZH2 by sponging miR-150-5p. *Cell Death & Disease* 12: 1097. https://doi.org/10.1038/s41419-021-04386-0

Guo, T., Li, J., Zhang, L., Hou, W., Wang, R., Zhang, J. & Gao, P. 2019. Multidimensional communication of microRNAs and long non-coding RNAs in lung cancer. *Journal of Cancer Research and Clinical Oncology* 145(1): 31-48. https://doi.org/10.1007/s00432-018-2767-5

Hao, S., Li, F., Li, S., Li, Q., Liu, Y., Yang, Q., Ye, X. & Wang, C. 2022. miR-3150a-3p, miR-6883-3p and miR-627-5p participate in the phycocyanin-mediated growth diminishment of A549 cells, via regulating a common target toll/interleukin 1 receptor domain-containing adaptor protein. *Journal of Functional Foods* 91: 105011. https://doi.org/10.1016/j.jff.2022.105011

Hao, S., Yang, Q., Li, F., Li, Q., Liu, Y., Li, S., Zhao, L. & Wang, C. 2021. Dysregulated expression of miR-642a-5p and its target receptor-interacting serine/threonine-protein kinase 1 contribute to the phycocyanin-mediated inhibitory function on non-small cell lung cancer. *Journal of Functional Foods* 85: 104654. https://doi.org/10.1016/j.jff.2021.104654

Hao, S., Li, S., Wang, J., Zhao, L., Yan, Y., Cao, Q., Wu, T.T., Liu, L.Y. & Wang, C.T. 2018. Transcriptome analysis of phycocyanin-mediated inhibitory functions on non-small cell lung cancer A549 cell growth. *Marine Drugs* 16(12): 511. https://doi.org/10.3390/md16120511

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R. & Salzberg, S-L. 2013. TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 14(4): R36. http://genomebiology.com/2013/14/4/R36

Huang, J.W., Luo, X.Y., Li, Z.H. & Lang, B.P. 2020. LncRNA NNT-AS1 regulates the progression of lung cancer through the NNT-AS1/miR-3666/E2F2 axis. *European Review for Medical and Pharmacological Sciences* 24(1): 238-248. https://doi.org/10.26355/eurrev_202001_19916

Khandelwal, A., Bacolla, A., Vasquez, K.M. & Jain, A. 2015. Long non-coding RNA: A new paradigm for lung cancer. *Molecular Carcinogenesis* 54(11): 1235-1251. https://doi.org/10.1002/mc.22362

Li, B., Gao, M-H., Chu, X-M., Teng, L., Lv, C-Y., Yang, P. & Yin, Q-F. 2015. The synergistic antitumor effects of all-trans retinoic acid and C-phycocyanin on the lung cancer A549 cells *in vitro* and *in vivo. European Journal of Pharmacology* 749: 107-114. https://doi.org/10.1016/j.ejphar.2015.01.009

Li, B., Gao, M-H., Lv, C-Y., Yang, P. & Yin, Q-F. 2016. Study of the synergistic effects of all-transretinoic acid and C-phycocyanin on the growth and apoptosis of A549 cells. *European Journal of Cancer Prevention* 25(2): 97-101. https://doi.org/10.1097/cej.0000000000000157

Liu, Q., Huang, Y., Zhang, R., Cai, T. & Cai, Y. 2016. Medical application of *Spirulina platensis* derived C-phycocyanin. *Evidence-Based Complementary and Alternative Medicine* 2016: 7803846. https://doi.org/10.1155/2016/7803846

Loewen, G., Jayawickramarajah, J., Zhuo, Y. & Shan, B. 2014. Functions of lncRNA HOTAIR in lung cancer. *Journal of Hematology & Oncology* 7: 90. https://doi.org/10.1186/s13045-014-0090-4

Luo, D.B., Lv, H.B., Sun, X.H., Wang, Y., Chu, J.H. & Salai, A.L. 2020. LncRNA TRERNA1 promotes malignant progression of NSCLC through targeting FOXL1. *European Review for Medical and Pharmacological Sciences* 24(3): 1233-1242. https://doi.org/10.26355/eurrev_202002_20176

Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S. & Baradaran, B. 2017. The different mechanisms of cancer drug resistance: A brief review. *Advanced Pharmaceutical Bulletin* 7(3): 339-348. https://doi.org/10.15171/apb.2017.041

Marcela Herrera-Solorio, A., Peralta-Arrieta, I., Armas Lopez, L., Hernandez-Cigala, N., Mendoza Milla, C., Ortiz Quintero, B., Catalan Cardenas, R., Pineda Villegas, P., Rodriguez Villanueva, E., Trejo Iriarte, C.G., Zuniga, J., Arrieta, O. & Avila-Moreno, F. 2021. LncRNA SOX2-OT regulates AKT/ERK and SOX2/GLI-1 expression, hinders therapy, and worsens clinical prognosis in malignant lung diseases. *Molecular Oncology* 15(4): 1110-1129. https://doi.org/10.1002/1878-0261.12875

Molina, J.R., Yang, P.G., Cassivi, S.D., Schild, S.E. & Adjei, A.A. 2008. Non-small cell lung cancer: Epidemiology, risk factors, treatment, and survivorship. *Mayo Clinic Proceedings* 83(5): 584-594. https://doi.org/10.4065/83.5.584

Morgenstern, D., Barzilay, R. & Levin, Y. 2021. RawBeans: A simple, vendor-independent, raw-data quality-control tool. *J. Proteome Res.* 20(4): 2098-2104. https://dx.doi.org/10.1021/acs.jproteome.0c00956

Nian, Y., Hu, X., Zhang, R., Feng, J., Du, J., Li, F., Bu, L., Zhang, Y., Chen, Y. & Tao, C. 2022. Mining on Alzheimer's diseases related knowledge graph to identity potential AD-related semantic triples for drug repurposing. *BMC Bioinformatics* 23(Suppl 6): 407. https://doi.org/10.1186/s12859-022-04934-1

Novikova, I.V., Hennelly, S.P. & Sanbonmatsu, K.Y. 2013. Tackling structures of long noncoding RNAs. *International Journal of Molecular Sciences* 14(12): 23672-23684. https://doi.org/10.3390/ijms141223672

Qi, X., Zhang, D-H., Wu, N., Xiao, J-H., Wang, X. & Ma, W. 2015. ceRNA in cancer: Possible functions and clinical implications. *Journal of Medical Genetics* 52(10): 710-718. https://doi.org/10.1136/jmedgenet-2015-103334

Ransohoff, J.D., Wei, Y. & Khavari, P.A. 2018. The functions and unique features of long intergenic non-coding RNA. *Nature Reviews Molecular Cell Biology* 19(3): 143-157. https://doi.org/10.1038/nrm.2017.104

Ren, C., Deng, M., Fan, Y., Yang, H., Zhang, G., Feng, X., Li, F., Wang, D., Wang, F. & Zhang, Y. 2017. Genome-wide analysis reveals extensive changes in LncRNAs during skeletal muscle development in Hu sheep. *Genes* 8(8): 191. https://doi.org/10.3390/genes8080191

Rezansoff, A.M., Laing, R., Martinelli, A., Stasiuk, S., Redman, E., Bartley, D., Holroyd, N., Devaney, E., Sargison, N.D., Doyle, S., Cotton, J.A. & Gilleard, J.S. 2019. The confounding effects of high genetic diversity on the determination and interpretation of differential gene expression analysis in the parasitic nematode *Haemonchus contortus. Int. J. Parasitol*. 49(11): 847-858. https://doi.org/10.1016/j.ijpara.2019.05.012

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. 2021. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* 71(3): 209-249. https://doi.org/10.3322/caac.21660

Taneja, S.B., Callahan, T.J., Paine, M.F., Kane-Gill, S.L., Kilicoglu, H., Joachimiak, M.P. & Boyce, R.D. 2023. Developing a knowledge graph for pharmacokinetic natural product-drug interactions. *J. Biomed. Inform.* 140: 104341. https://doi: 10.1016/j.jbi.2023.104341

Tuo, Z., Liang, L. & Zhou, R. 2021. LINC00852 is associated with poor prognosis in non-small cell lung cancer patients and its inhibition suppresses cancer cell proliferation and chemoresistance via the hsa-miR-145-5p/KLF4 axis. *Journal of Gene Medicine* 23(12): e3384. https://doi.org/10.1002/jgm.3384

Wan, Y., Yao, D., Fang, F., Wang, Y., Wu, G. & Qian, Y. 2021. LncRNA WT1-AS downregulates lncRNA UCA1 to suppress non-small cell lung cancer and predicts poor survival. *BMC Cancer* 21(1): 104. https://doi.org/10.1186/s12885-020-07767-4

Wu, X., Sui, Z., Zhang, H., Wang, Y. & Yu, Z. 2020. Integrated analysis of lncRNA-mediated ceRNA network in lung adenocarcinoma. *Frontiers in Oncology* 10: 554759. https://doi.org/10.3389/fonc.2020.554759

Yi, S., Li, G. & Sun, B. 2021. Overexpression of LINC00852 promotes prostate cancer cell proliferation and metastasis. *Asia-Pacific Journal of Clinical Oncology* 17(6): 435-441. https://doi.org/10.1111/ajco.13418

Zhang, G., Wang, Q., Zhang, X., Ding, Z. & Liu, R. 2019. LncRNA FENDRR suppresses the progression of NSCLC via regulating miR-761/TIMP2 axis. *Biomedicine & Pharmacotherapy* 118: 109309. https://doi.org/10.1016/j.biopha.2019.109309

Zhang, X., Chen, Z., Zang, J., Yao, C., Shi, J., Nie, R. & Wu, G. 2021. LncRNA-mRNA co-expression analysis discovered the diagnostic and prognostic biomarkers and potential therapeutic agents for myocardial infarction. *Aging-Us* 13(6): 8944-8959.

Zhang, Y.X., Yuan, J., Gao, Z.M. & Zhang, Z.G. 2018. LncRNA TUC338 promotes invasion of lung cancer by activating MAPK pathway. *European Review for Medical and Pharmacological Sciences* 22(2): 443-449.

Zhao, L., Zhang, X., Shi, Y. & Teng, T. 2020. LncRNA SNHG14 contributes to the progression of NSCLC through miR-206/G6PD pathway. *Thoracic Cancer* 11(5): 1202-1210. https://doi.org/10.1111/1759-7714.13374

Zhen, Q., Gao, L-N., Wang, R-F., Chu, W-W., Zhang, Y-X., Zhao, X-J., Lv, B-L. & Liu, J-B. 2018. LncRNA DANCR promotes lung cancer by sequestering miR-216a. *Cancer Control* 25(1): 1073274818769849. https://doi.org/10.1177/1073274818769849

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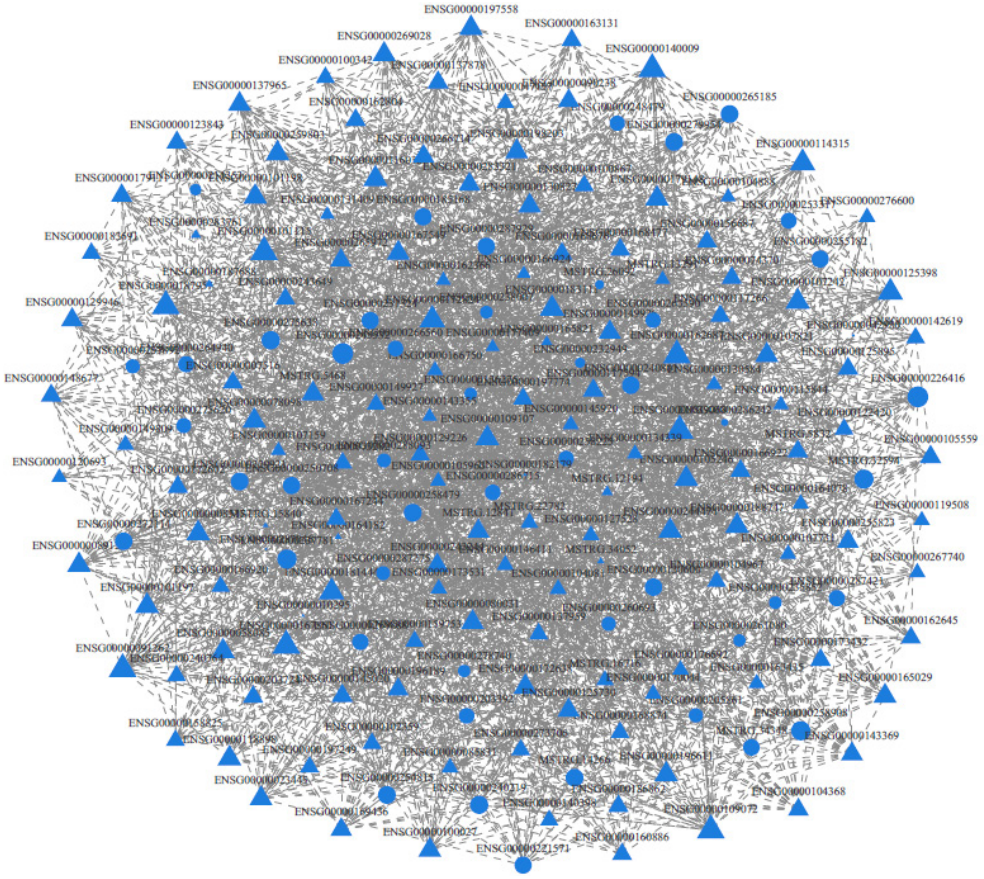

FIGURE S1. lncRNA-mRNA co-expression networks. Round nodes represent lncRNAs, and triangle nodes represent mRNAs. The lines between nodes represent interactions between two genes. Degree is defined as the numbers of links one node has to the other nodes

TABLE S1. Statistics of lncRNAs

Sample	Known lncRNA num	Novel lncRNA num	Total
Con-1	25,075	1,458	26,533
Con-2	24,543	1,464	26,007
Con-3	25,111	1,486	26,597
PC-1	25,334	1,468	26,802
PC-2	25,836	1,481	27,317
PC-3	25,738	1,483	27,221

TABLE S2. Classification and subgroup of lncRNAs

Type	Known lncRNA num	Novel lncRNA num	Total
antisense	17768	1224	18992
sense intron overlap	615	25	640
intergenic	36889	207	37096
sense exon overlap	6813	362	7175
bidirection	5147	2	5149

TABLE S3. The top expressed 15 lncRNA transcripts in Con and PC groups

Con		PC	
lncRNAs	TPM	lncRNAs	TPM
ENST00000602361	58511.15	ENST00000602361	64810.17
ENST00000554988	16530.64	ENST00000554988	14324.52
ENST00000618589	8645.37	ENST00000618589	7671.39
NONHSAT243766.1	6137.81	NONHSAT243766.1	6700.25
ENST00000659240	4918.28	ENST00000659240	5387.17
NONHSAT052027.2	2032.83	NONHSAT135828.2	2919.21
NONHSAT146442.2	1884.87	ENST00000610851	2154.29
ENST00000618227	1408.33	ENST00000618227	2010.76
ENST00000616691	1106.53	ENST00000616691	1871.71
NONHSAT021728.2	1076.28	NONHSAT052027.2	1701.58
ENST00000610851	1071.01	ENST00000508832	1288.08
ENST00000541782	994.52	ENST00000534336	1157.87
NONHSAT190639.1	910.08	NONHSAT021728.2	977.59
ENST00000534336	625.55	ENST00000541782	838.71
NONHSAT176665.1	594.18	NONHSAT146442.2	707.9

TABLE S4. Top 10 upregulated and 10 downregulated lncRNA transcripts

LncRNA ID	Log_2FC(PC/Con)	Regulate
NONHSAT054508.2	10.44	Up
ENST00000654005	9.14	Up
NONHSAT234256.1	9.11	Up
NONHSAT104167.2	8.76	Up
NONHSAT135554.2	8.18	Up
NONHSAT216908.1	7.51	Up
ENST00000670471	7.5	Up
NONHSAT200775.1	7.42	Up
NONHSAT249575.1	7.4	Up
ENST00000660542	7.4	Up
ENST00000538717	-10.01	Down
NR_152799.1	-9.64	Down
NONHSAT183309.1	-8.34	Down
ENST00000488584	-8.14	Down
ENST00000657910	-8.13	Down
NONHSAT257741.1	-7.93	Down
NONHSAT243455.1	-7.73	Down
NONHSAT242091.1	-7.67	Down
NONHSAT031960.2	-7.49	Down
NONHSAT239845.1	-7.37	Down