Original Article

Analysis of the expression profile of serum exosomal lncRNA in breast cancer patients

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Background: Breast cancer (BC) is a common tumor that seriously affects women's physical/mental health and even life. BC invasion and metastasis are still the main causes of mortality in BC patients. Exosomal long non-coding RNAs (exo-lncRNA) play an important role in cell communication and can help to understand better the physiological and pathological conditions that result from BC. This study investigates new potential targets and functions of the expression profiles of exo-lncRNAs in BC patients through high-throughput screening and bioinformatics.

Methods: Samples were collected from two BC patients and one healthy subject. The serum exosomal RNAs were subsequently purified, and a library was established for quality inspection and sequencing. The resultant data was compared with the reference data to obtain the differential expression of exo-lncRNAs, and predict the target genes. To obtain the final results, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to annotate the function and pathway of the differentially expressed genes.

Results: After a comprehensive comparison of the BC patients and healthy subjects, we discovered five up-regulated exo-lncRNAs and six down-regulated exo-lncRNAs of interest. Combining our results with a literature review and screening, we found that *VIM-AS1*, *SNHG8*, and *ELDR* play a role in the progression of BC, with *VIM-AS1* predicting 35 target miRNAs; *SNHG8* predicting 12 target miRNAs, and *ELDR* predicting 24 target miRNAs. Target prediction considered that the target gene of *VIM-AS1* was *VIM* and that the target gene of *SNHG8* was *PRSS12*. GO enrichment analysis showed that *VIM* mainly played a role in cell processes, biological regulation, metabolic regulation, and molecular adhesion, while *PRSS12* was enriched through cell metabolism, catalytic activity, and hydrolase activity. KEGG pathway enrichment results also indicated how the *VIM* protein functions in cancer development through the viral infection signaling pathway and miRNA signaling pathway.

Conclusions: There is a significant difference in the expression profiles of serum exo-lncRNAs between BC patients and healthy individuals. This may be closely related to BC's occurrence, development, and metastasis, and therefore provides a theoretical basis for more in-depth studies into exo-lncRNA.

Keywords: Breast cancer (BC); exosomes; long non-coding RNA; biomarkers; tumor

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Introduction

Breast cancer (BC) is the most widely diagnosed cancer among women and is the most fatal malignant tumor type, with a morbidity and mortality rate of 25% and 15%, respectively (1). According to the latest data, more than 250,000 patients are diagnosed with BC every year in the United States (2). Although 70% of genes can be transcribed into ribonucleic acids (RNAs), only 2% of these transcripts have been translated into proteins in the human genome. Other transcripts are defined as non-coding RNA and can be divided into two categories. The first is short-chain non-coding RNAs (sncRNAs), which include microRNAs (miRNAs), small interfering RNAs (siRNAs), and small nucleolar RNAs (snoRNAs). The second category includes long-chain non-coding RNAs (lncRNAs) (3). sncRNAs are mainly considered a negative regulator of gene expression, while lncRNAs have been identified as a widely heterogeneous population, and studies of their gene expression have attributed them to the development of many human diseases, including cancer (4,5). Exosomes are membrane vesicles with a diameter of 20–200 nm. They belong to the larger family of extracellular vehicles (EVs) produced in cells and released into the extracellular space (6). Many studies have shown that exosomes are the key mediator of intercellular communication between tumor and stromal cells in local and distant microenvironments (7). Exosomes derived from tumors promote angiogenesis and coagulation, regulate the immune system, reshape the surrounding parenchyma, and jointly support tumor progression (8,9). These exosomes participate in many cellular functions and are considered important cellular communication connectors as they contain various proteins and nucleic acids, including miRNAs and lncRNAs (10,11).

In line with increasing research on the topic, a large amount of evidence shows that the signals transmitted by exo-lncRNAs regulate many types of local and distant receptor cells. In addition, exosome-mediated lncRNA transfer pathways are different in level and type under normal physiological and pathological conditions. Exosomes are extracellular vesicles secreted by different types of cells. Exosomes have become an indispensable promoter of information exchange between cells. More importantly, exosomes play a vital role in various diseases including cancer. Exosomes lncRNAs play a central role in carcinogenesis and cancer progression by regulating tumor growth, metastasis, angiogenesis and chemotherapy resistance. In addition, exosomes lncRNAs play a messenger role in intercellular communication, thus remodeling tumor microenvironment. Their functional relevance in cancer biology suggests that exosome lncRNA may be a promising non-invasive biomarker in future cancer treatment.We used Exo-lncRNA chip technology to detect and compare the differential expression profiles of serum exo-lncRNAs between BC patients and healthy subjects for this study. This was done by screening out the differential exo-lncRNAs and analyzing their targets/possible functional mechanisms through bioinformatics. Through this approach, we hoped to further research on the early diagnosis and treatment of BC. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-3483).

Methods

General information

Blood samples were collected from two patients who were diagnosed for the first time with BC at Henan Cancer Hospital (from October 2019 to December 2019). One had early-stage BC (T1), while the other had advanced BC (M1). The pathological type of both patient tumors was confirmed as non-special invasive BC (NST) by a pathologist (T1: early-stage NST; M1: advanced BC with bone metastasis). A further blood sample was collected from a healthy female volunteer. No clinical treatment was given to either BC patient before sample collection. The study was undertaken following the World Medical Association (WMA) Declaration of Helsinki (as revised in 2013), the National Health and Family Planning Commission's Measures for the Ethical Review of Biomedical Research Involving Human Beings, and other relevant laws and regulations. It was approved by the Medical Ethics Committee of Henan Cancer Hospital. All the subjects gave their consent and signed the relevant consent form.

Instruments and main reagents

Instrument: Agilent 2200 TapeStation Software (Agilent Technologies, CA, USA); Reagents: Qubit (Life Technologies, MA, USA); RNA ScreenTape and RNA Reagent (Agilent Technologies, CA, USA); D1000 ScreenTape and D1000 Reagent (Agilent Technologies, CA, USA); RiboTM Exosome Isolation Reagent (RiboBio, Guangzhou, China); Qubit dsDNA HS Assay Kit (Life Technologies, MA, USA); Agencourt Ampure XP beads (Beckman Coulter, CA, USA);

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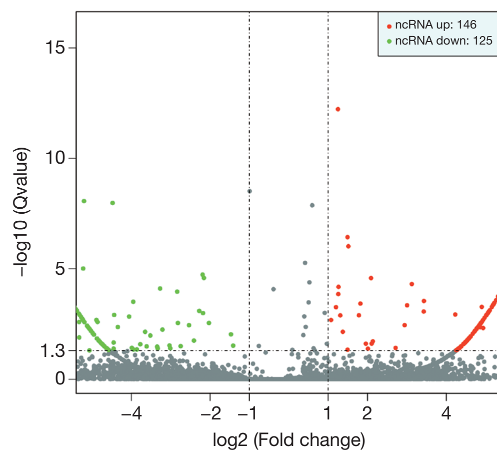

Figure 1 Volcanic map of the exosomal lncRNAs with differential expression.

NEB NEXT Ultra RNA Library Prepkit for Illumina (New England Biolabs, MA, USA).

Extraction and identification of serum exosomes

Peripheral venous blood was collected from the subjects with an EDTA-K2 anticoagulation tube. After collection, the sample remained still before being centrifuged. The serum was then collected and packed in sterile EP tubes and temporarily stored at −80 ℃. Exosomes were identified by particle size detection and marker protein analysis, and the antibodies used included CD9, CD63, TSGl01, and goat anti-rabbit secondary antibody.

Exosomal RNA extraction and exosomal lncRNA high-throughput sequencing

Exosomal RNAs were extracted following the relevant step-by-step instructions. RNAs in serum exosomes were extracted, and samples were tested by Qubit and Agilent 2200 TapeStation. We then constructed the library following these key steps: (I) RNA fragmentation; (II) first-chain complementary DNA (cDNA) synthesis; (III) synthesis and purification of second-chain cDNA; (IV) end repair and detailing; (V) adapter connection, fragment selection, and purification; (VI) polymerase chain reaction (PCR) amplification and purification. Using the Agilent 2200 TapeStation, the samples then passed a library-quality inspection. This involved preparing the on-machine samples according to the method described in the HiSeq User Guide. Finally, the sequencing was carried out by the Guangzhou Ribobio Biological Company, and the expression data of lncRNAs were analyzed by comparing them with the known gene sequences.

Differential screening

The expression difference between exo-lncRNAs was screened and identified using the following steps: (I) the gene type was set as lncRNA; (II) the expression multiple [log2(Fold change), expression difference multiple] of exosome lncRNAs in the BC group was found to be greater than or equal to 1, or less than or equal to 1.5; (III) exocrine lncRNAs with either high or low expressions were identified in both samples provided by the T1 and M1 BC patients.

Bioinformatics and statistical analysis

Cis/trans target genes were identified in the lncRNAs, and their enrichment functions and signal pathways were analyzed through GO and KEGG analysis. This helped us to predict further the cellular function and signal pathways involved in these abnormally expressed lncRNAs, as well as speculate on the biological function of the lncRNAs. In the prediction of lncRNA-miRNA, miRanda, PITA, and RNAhybrid were used to establish the recognition areas of lncRNA and miRNA.

Results

Exo-lncRNA sequencing results

The statistical results of the screening of the differentially expressed exo-lncRNAs are shown in *Figure 1*. We found that the BC patients had 146 exo-lncRNAs up-regulated and 125 exo-lncRNAs down-regulated when compared with the healthy subject.

The most significantly differentially expressed exo-lncRNAs and a literature review

Through comprehensively comparing the healthy subject with the T1 and M1 BC patients, we obtained 11 significantly different Exo-lncRNAs according to the screening principle. Five of these exosomes displayed a significant increase in the expression levels of lncRNAs, while the other 6 exemplified a significant decrease in expression levels (*Table 1*). A literature review was performed on these 11 exo-lncRNAs, and 3 of them were identified as *VIM-AS1*, *SNHG8*, and *ELDR*, all of which

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Table 1 The exosomal lncRNAs with significantly different expressions

Lnc-RNA	Gene	Type	Log2 (fold change)	P
NR_135133.1	*LOC101928932*	lncRNA	4.440055	0.00041
NR_102754.1	*SPATA8-AS1*	lncRNA	4.369055	0.000587
NR_144487.1	*CHL1-AS2*	lncRNA	4.25746	0.001
NR_051989.1	*LINC00534*	lncRNA	2.135252	0.000289
NR_108060.1	*VIM-AS1*	lncRNA	1.258204	8.34E-07
NR_121667.1	*LOC101927854*	lncRNA	−1.54641	7.07E-24
NR_034010.1	*SNHG8*	lncRNA	−3.20761	7.01E-05
NR_125380.1	*LOC100288798*	lncRNA	−3.2679	4.17E-07
NR_110426.1	*ELDR*	lncRNA	−4.4345	1.11E-05
NR_110278.1	*LOC101927881*	lncRNA	−5.91017	0.000987
NR_135057.1	*LOC105376805*	lncRNA	−6.71971	0.000745

Table 2 Candidate lncRNA cis and trans target prediction results and detailed information

Transcript	Cis-prediction	Trans-prediction	lncRNA	Chromosome	mRNA	Gene
NR_108060.1	1	0	VIM-AS1	chr10	NM_003380.3	VIM
NR_034010.1	1	0	SNHG8	chr4	NM_003619.3	PRSS12
NR_110426.1	0	0	ELDR	–	–	–

we further analyzed. *VIM-AS1* was noted as up-regulated, while *SNHG8* and *ELDR* were down-regulated.

Prediction and analysis of lncRNA targets

The prediction and detailed information of cis and trans lncRNA targets can be seen in *Table 2*. The target gene vim of *VIM-AS1* was predicted in cis, and the target gene *PRSS12* of *SNHG8*. As for the lncRNA-miRNA prediction analysis target, the results were drawn from three different types of software (miRanda, PITA, and RNAhybrid) and were further screened. The results of which looked at the interaction of the miRNAs in the lncRNAs, with *VIM-AS1* predicting 35 target miRNAs, *SNHG8* was predicting 12 target miRNAs, and *ELDR* predicting 24 target miRNAs. *Figures 2-4* show the software results.

Analysis of GO saliency function enrichment and KEGG pathway saliency enrichment

The functional and signaling pathway enrichment analyses of the GO and KEGG target genes in the lncRNA candidate revealed that *VIM*, the target gene of *VIM-AS1*, was enriched with 107 functions in biological processes such as cellular processes, biological regulation, metabolic regulation, macromolecular metabolic processes, stimulation reactions, and others. There were 43 functions enriched in the cell components, which were mainly concentrated in the cells and cytoplasm. In terms of the molecular function, 19 functions were enriched, including adhesion, protein, macromolecule, binding, organic compound, and the complex combination of nucleic acid and protein. *PRSS12*, a target gene of *SNHG8*, was biologically enriched with 24 functions, including cellular metabolism, organic metabolism, macromolecular metabolism, gene expression, and protein metabolism. 30 functions were enriched in cell components, mainly concentrated in the cells, cytoplasm, cell membrane, and cell periphery. 12 functions were also enriched in the molecular function; this mainly included catalytic activity, hydrolase activity, receptor activity, molecular sensor, endopeptidase activity, and serine hydrolase

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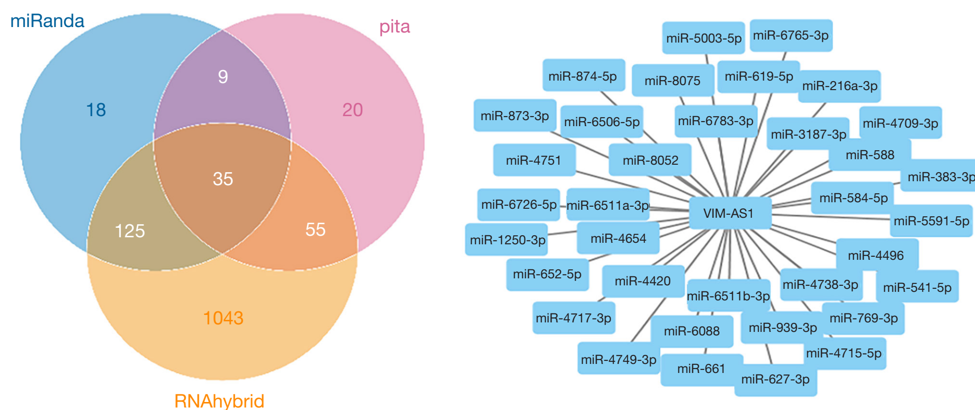

Figure 2 Predicted target miRNA of VIM-AS1.

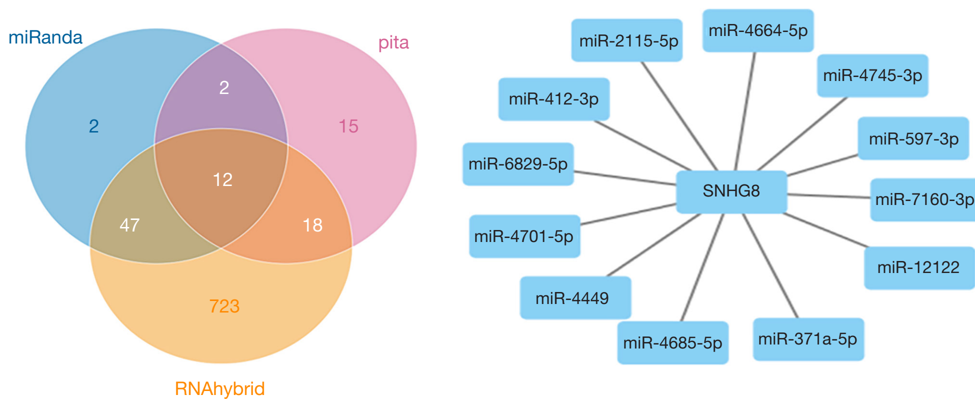

Figure 3 Predicted target miRNA of SNHG8.

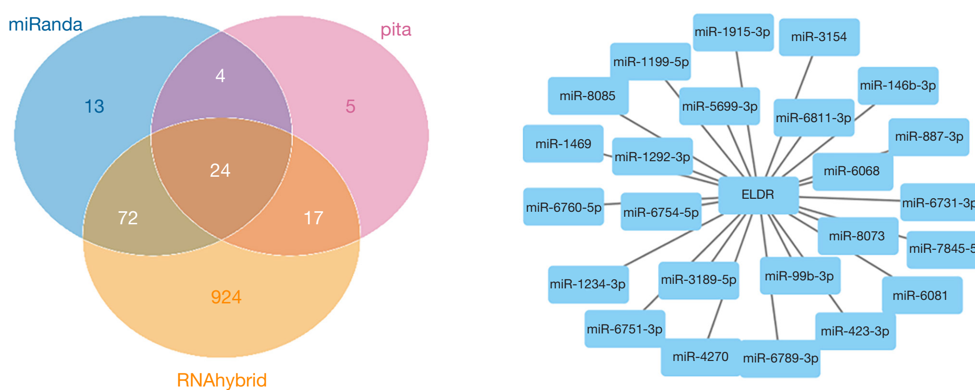

Figure 4 Predicted target miRNA of ELDR.

activity. KEGG pathway enrichment results also indicated how the *VIM* protein functions in cancer development through the viral infection signaling pathway and miRNA signaling pathway. The top 10 functions were listed based on the database sample size compared by GO analysis (*Tables 3-5*).

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Table 3 Top ten enrichment functions of VIM

GO-ID	Term
Biological process	
GO:0009987	Cellular process
GO:0044699	Single-organism process
GO:0044763	Single-organism cellular process
GO:0065007	Biological regulation
GO:0008152	Metabolic process
GO:0050789	Regulation of biological process
GO:0050794	Regulation of cellular process
GO:0043170	Macromolecule metabolic process
GO:0050896	Response to stimulus
GO:0032501	Multicellular organismal process
Cellular component	
GO:0044464	Cell part
GO:0005623	Cell
GO:0005622	Intracellular
GO:0044424	Intracellular part
GO:0043226	Organelle
GO:0043229	Intracellular organelle
GO:0043227	Membrane-bounded organelle
GO:0043231	Intracellular membrane-bounded organelle
GO:0005737	Cytoplasm
GO:0044444	Cytoplasmic part
Molecular function	
GO:0005488	Binding
GO:0005515	Protein binding
GO:0097159	Organic cyclic compound binding
GO:1901363	Heterocyclic compound binding
GO:0003676	Nucleic acid binding
GO:0097367	Carbohydrate derivative binding
GO:0003723	RNA binding
GO:0044877	Macromolecular complex binding
GO:0042802	Identical protein binding
GO:0032403	Protein complex binding

Table 4 Top ten enrichment functions of PRSS12

GOID	Term
Biological process	
GO:0009987	Cellular process
GO:0044699	Single-organism process
GO:0008152	Metabolic process
GO:0044763	Single-organism cellular process
GO:0071704	Organic substance metabolic process
GO:0044238	Primary metabolic process
GO:0043170	Macromolecule metabolic process
GO:0051179	Localization
GO:0010467	Gene expression
GO:0019538	Protein metabolic process
Cellular component	
GO:0044464	Cell part
GO:0005623	Cell
GO:0005622	Intracellular
GO:0044424	Intracellular part
GO:0043226	Organelle
GO:0043227	Membrane-bounded organelle
GO:0005737	Cytoplasm
GO:0016020	Membrane
GO:0044444	Cytoplasmic part
GO:0071944	Cell periphery
Molecular function	
GO:0003824	Catalytic activity
GO:0016787	Hydrolase activity
GO:0004872	Receptor activity
GO:0060089	Molecular transducer activity
GO:0070011	Peptidase activity, acting on L-amino acid peptides
GO:0008233	Peptidase activity
GO:0004175	Endopeptidase activity
GO:0008236	Serine-type peptidase activity
GO:0017171	Serine hydrolase activity
GO:0004252	Serine-type endopeptidase activity

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Table 5 Signal pathways enriched in VIM

Signal pathway	ID
Epstein-Barr virus infection	hsa05169
MicroRNAs in cancer	hsa05206

Discussion

Exosomes are bilayer lipid membrane vesicles with a diameter of about 30–100nm. They contain a variety of protein and nucleic acid components without organelles. They are cup-shaped under electron microscopy and generally have a spherical structure in body fluid. The density range is 1.13–1.19 g/mL (12) in a sucrose density gradient solution. Studies have shown that when compared with healthy subjects, the concentration of exosomes in the plasma of BC patients is significantly increased. This indicates that the number of exosomes in plasma can aid the identification of BC (13). Ewaisha *et al.* (14) also found that the plasma exosomes of BC patients express specific proteins and RNAs, which play a major role in the occurrence and development of BC. This only further indicates that the contents of exosomes have the potential to be biomarkers for diagnosing BC.

Miao *et al.* (15) found that the expression level of lncRNA *MALATl* in the cancer tissue of BC patients was significantly higher than that in the para-cancerous tissue. They also found that the expression level of *MALATl* in serum samples of BC patients was significantly higher than that in benign breast diseases. In addition, scientists found that lncRNA *H19* was significantly expressed in BC serum samples and found that its expression in serum samples after surgery was significantly lower than serum samples taken before surgery. All this research indicates that lncRNAs can be used for the differential diagnosis of BC and has a certain value in the context of prognosis detection. Through a further comparison of the BC patients and the healthy subject, lncRNAs with traditional biomarkers such as CEA and CA-l53 also revealed that lncRNAs had high sensitivity and specificity, even exceeding the sensitivity of traditional ultrasonic diagnostic methods (16). lncRNAs can also be packaged into exosomes and act as messengers in intercellular communication, indicating the further potential for using lncRNAs as a diagnostic and prognostic marker for various other cancers (17,18). The exosome lncRNA Xist-mediated pathway is activated in the early stage of metastatic BC in the brain and may become an effective target for treating brain metastasis (19). The expression of exosome *HOTAIR* is positively correlated with the state of the receptor tyrosine kinase (RTK) ErbB2 (also known as HER2/neu) in tumor tissues and suggests poor prognosis and chemotherapeutic efficacy (20,21). Exosome H19 can be used as a biomarker for predicting BC and can induce BC resistance (22,23).

VIM-AS1 is a 1.8-kb non-coding RNA. Studies have shown that *VIM-AS1* has a hybrid R-loop structure of DNA, shares a bidirectional promoter transcription with *VIM* mRNA, and can positively regulate Vim expression (24). This is consistent with our predicted target results, but further verification is needed. *VIM-AS1* has also been confirmed in human colon cancer cell lines, with its expression being closely related to tumor progression and found to be significantly up-regulated with the progression of tumors. More specifically, the expression of *VIM-AS1* in human colon cancer cell lines was up-regulated in lymph node metastasis and vascular invasion tumors. Subsequent *in vitro* experiments proved that *VIM-AS1* played a key role in promoting migration and EMT of colon cancer cells (25), thus further demonstrating the importance of *VIM-AS1* in tumorigenesis. The second important exo-lncRNA from our study was *SNHG8*, which has been noted to play the role of an oncogene in many kinds of tumors, and which was also consistent with our sequencing results. *SNHG8* is also involved in tumor drug resistance, angiogenesis, and epithelial-mesenchymal transition, which is consistent with our target prediction and functional analysis. Recent studies have also suggested that a targeted *ELDR* injection has good therapeutic potential for oral cancer (26). From our findings, we believe that the role lncRNAs play in BC still requires further attention.

Nevertheless, from analyzing the functions of *VIM-AS1*, *SNHG8*, and *ELDR* genes in tumor genesis and development, we believe these three key genes screened are deserving of further research. To conclude, this study enriched the differential expression profile of serum exosomes of BC patients, searched for and identified the target genes of candidate lncRNAs through bioinformatic methods, and predicted their functions and related signaling pathways. This has ultimately provided more reasons for further research on the role and mechanism of lncRNAs in BC. In addition, due to the small sample size of this study, future research on the role of lncRNAs should be expanded further to verify the appropriateness of our lncRNA candidate selection. This could be carried out by using real-time fluorescence quantitative PCR technology and molecular biology to verify and clarify the biological functions of lncRNAs in the

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development and occurrence of tumors.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-3483). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was undertaken following the World Medical Association (WMA) Declaration of Helsinki (as revised in 2013), the National Health and Family Planning Commission's Measures for the Ethical Review of Biomedical Research Involving Human Beings, and other relevant laws and regulations. It was approved by the Medical Ethics Committee of Henan Cancer Hospital (V1.0-20191029). All the subjects gave their consent and signed the relevant consent form.

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