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Journal of Advanced Research in Health Sciences
Sağlık Bilimlerinde İleri Araştırmalar Dergisi,
JARHS 2025, 8 (2): 69–75

https://doi.org/10.26650/JARHS2025-1608689

Submitted: 29.12.2024
Revision Requested: 11.02.2025
Last Revision Received: 16.03.2025
Accepted: 17.03.2025
Published Online 09.05.2025

Journal of Advanced Research in Health Sciences
Sağlık Bilimlerinde İleri Araştırmalar Dergisi

Research Article

Open Access

Determining the Expression Levels of Lncrna PVT1 and Mir-128 in the Human Lung Carcinoma Cell Line

İnsan Akciğer Karsinomu Hücre Hattında Lncrna PVT1 ve Mir-128'in İfade Düzeylerinin Belirlenmesi

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Abstract

Objective: In the presented research, we aimed to examine the expression of microRNA-128 (miR-128) and long non-coding RNA (lncRNA) the plasmacytoma variant translocation 1 (PVT1) in non-cancerous cells BEAS-2B and human lung carcinoma A549 to identify any potential roles these molecules might play in the cancer development in lung tissue.

Material and Methods: The cell lines used in this research were A549 and BEAS-2B. Total RNA was isolated from these cell lines, and then complementary DNA (cDNA) was synthesised. Using the quantitative real-time PCR (RT-qPCR) method, the expression levels of lncRNA PVT1 and miR-128 were evaluated. The $2^{-\Delta\Delta Ct}$ method was employed to analyse fold changes by normalising to U6 snRNA and GAPDH expressions.

Results: Elevated PVT1 expression was determined in the lung cancer cells ($p<0.01$). Nevertheless, miR-128 expression did not change statistically significantly ($p>0.05$). Even though miR-128 is a potential binding target of lncRNA PVT1 according to in silico analysis, the expression profile does not correlate.

Conclusion: Our evaluation of the relationship between lncRNA PVT1 and miR-128 in cancerous cells A549 compared with non-cancerous cells BEAS-2B reveals that PVT1 expression is significantly

Öz

Amaç: Araştırmamızın amacı, normal akciğer epitel hücre hatlarında (BEAS-2B) ve insan akciğer karsinomunda (A549), mikroRNA-128 (miR-128) ve uzun kodlamayan RNA (lncRNA) PVT1 ekspresyonunu inceleyerek, bu moleküllerin akciğer kanserinin gelişimi ve ilerlemesinde oynayabileceği potansiyel rolleri belirlemektir.

Gereç ve Yöntemler: Bu araştırmada kullanılan hücre hatları A549 ve BEAS-2B'dir. Gerçek zamanlı kantitatif PCR (RT-qPCR) yöntemi kullanılarak PVT1 ve miR-128'in ifade seviyeleri değerlendirildi. $2^{-\Delta\Delta Ct}$ yöntemi, U6 snRNA ve GAPDH ifadelerine göre normalize ederek kat değişimlerini incelemek için kullanıldı.

Bulgular: Akciğer kanseri hücrelerinde PVT1 ekspresyonunun yükseldiği belirlendi ($p<0,01$). Bununla birlikte, miR-128 ekspresyonu istatistiksel olarak anlamlı bir şekilde değişmedi ($p>0,05$). Mir-128, in-silico analizde lncRNA PVT1'in potansiyel bir bağlanma hedefi olmasına rağmen, ekspresyon profili sonuçlarına göre korelasyon görülmedi.

Sonuç: Normal akciğer epitel hücrelerine kıyasla akciğer kanseri hücrelerinde lncRNA PVT1 ve miR-128 arasındaki ilişkinin incelendiği çalışmamız, PVT1 ekspresyonunun akciğer kanseri hücrelerinde önemli ölçüde yüksek olduğunu, buna karşın

Citation: Yenilmez Tunoğlu EN, Öter GN, Akar RO, Ulukaya E, Tanrıkulu Küçük S, Karpuzoğlu FH. Determining the expression levels of lncrna PVT1 and Mir-128 in the human lung carcinoma cell line. Journal of Advanced Research in Health Sciences 2025;8(2):69-75. https://doi.org/10.26650/JARHS2025-1608689

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elevated in lung cancer cells, whereas miR-128 expression remains unchanged. Further investigation is needed to explore whether the interaction between lncRNA PVT1 and miR-128 is indirect.

Keywords Lung cancer · A549 cell line · PVT1 · miR-128 · RT-qPCR

miR-128 ekspresyonunun değişmeden kaldığını ortaya koymaktadır. LncRNA PVT1 ve miR-128 arasındaki etkileşimin dolaylı olup olmadığını keşfetmek için daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler Akciğer kanseri · A549 hücre hattı · PVT1 · miR-128 · RT-qPCR

INTRODUCTION

Lung cancer (LC) is a prominent cause of cancer-associated mortality on a global scale. Each year, it affects 1.8 million individuals worldwide, resulting in 1.6 million deaths. Lung cancer is divided into two main subtypes. The first is small cell lung cancer (SCLC), which is present in 15% of new cases. Second is non-small-cell lung cancer (NSCLC), which represents about 85% of all newly diagnosed lung cancers (1). Challenges associated with detecting LC at an early stage persist because of the inadequacy of practical diagnostic tools and methods (2). Therefore, we need to explore new molecular markers and methodologies to enhance our elucidation of the molecular mechanisms behind this disease.

MicroRNAs (miRNAs) represent a distinct class of endogenous, small non-coding RNAs ranging from 18 to 27 nucleotides in length. They are essential for post-transcriptional gene regulation via attachment to the 3′ untranslated region (UTR) of specific mRNAs. They also participate in a broad spectrum of cellular activities, such as cell proliferation, metabolic processes, and programmed cell death. miRNAs have been recognised as key regulators in various cancer types, particularly through alterations in their expression profiles that influence cancer pathogenesis. Emerging evidence underscores the significant dysregulation of these non-coding RNA types in various cancers. In tumour tissues, specific miRNAs (OncomiRs) are overexpressed, whereas others (tumour suppressor miRNAs) are underexpressed. Reduced levels of tumour suppressor miRNAs and increased expression of oncomiRs contribute to key cancer hallmarks such as sustained proliferation, evasion of cell death mechanisms, evasion of immune surveillance, invasion, and metastasis. Differential expression of miRNAs has been observed in tumour tissues of patients with LC (3-5).

lncRNAs do not possess open reading frames (ORFs) and thus cannot encode proteins. The intricate functions of lncRNAs in cellular processes involve complex interactions with biological molecules such as DNA, RNA, proteins, and other cellular components. lncRNAs interact with RNA-binding proteins and can modulate transcription factors, activating or suppressing target genes at the post-transcriptional or post-translational levels. Recent investigations have underscored their pivotal involvement in tumorigenesis, functioning as oncogenes or tumour suppressors. They are intricately involved in critical biological processes such as cellular proliferation, apoptosis evasion, migration, and invasion. Consequently, research on lncRNAs holds substantial promise in elucidating the mechanisms underlying tumour initiation and progression (6-8).

An important aspect is that miRNAs and lncRNAs are capable of interacting with each other, creating intricate regulatory networks within cells. For instance, miRNAs can target and regulate the stability and abundance of lncRNAs. Conversely, lncRNAs can function as molecular sponges or competitive decoys for miRNAs, modulating their activity by sequestering them away from their mRNA targets. Moreover, miRNAs and lncRNAs can cooperatively regulate common target genes, enhancing the complexity and precision of gene expression control (9).

PVT1 is a key oncogenic factor in the progression of various malignancies. It operates as a competing endogenous RNA (ceRNA), modulating miRNA activity by serving as a molecular sponge. It binds to miRNAs, hindering their interaction with target mRNAs. However, this sponging effect of PVT1 is disrupted under cancerous conditions (10, 11). miR-128 is recognised as a prominent tumour suppressor that inhibits tumour growth, migration, and metastasis by downregulating oncogenic processes. miR-128 is involved in a range of diseases and cellular mechanisms, including cell proliferation, epithelial-mesenchymal transition (EMT), tumorigenesis, and angiogenesis. Recent research indicates that lncRNA PVT1 promotes the progression of different cancer types by functioning as a sponge for miR-128 (12-14). Accordingly, this research aims to shed light on the potential roles of lncRNA PVT1 and miR-128 in lung cancer (LC) development and progression by examining their expression levels in cancerous cells compared with non-cancerous BEAS-2 B cells.

MATERIALS AND METHODS

Cell line and culture conditions

Incubation of the A549 cell line (ATCC CRM-CCL-185 ™) and the BEAS-2B cell line (ATCC CRL-3588 ™) were conducted at 37 °C, the ratio of CO_2 was 5% and humidity 95% in CO_2 incubator (Panasonic, MCO-170M-PE). The maintenance of both cell lines was conducted in a culture medium composed of RPMI-1640 (Gibco), with the incorporation of Foetal Bovine Serum (Gibco)

and penicillin–streptomycin (Gibco), at concentrations of 10% and 1%, respectively. Once the cells reached confluence, they were subcultured by adding a Trypsin-EDTA solution (Gibco), followed by centrifugation and re-suspension in fresh media.

Total RNA extraction and complementary DNA Synthesis

Total RNA extraction, including miRNA, was performed from all cells using miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen) following the instructions. The RNA purity and quality were evaluated with a spectrometric measurement (NanoDrop, Thermo) by measuring the A260/A280 and A260/A230 ratios. The synthesis of complementary DNA was performed by reverse transcription using an RT² First Strand Kit (Qiagen). Additionally, the miRCURY LNA RT Kit (Qiagen) was used to reverse transcribe for the miRNA experiments. The total RNA yield of all samples was equalised to 500 ng for cDNA synthesis. After reverse transcription, cDNA concentrations were spectrophotometrically evaluated to check the synthesis step. All cDNA concentrations were between 1400 ng/µL and 1450 ng/µL, and these were then used in the RT-qPCR experiment.

Real-time quantitative PCR

Commercially available and wet-lab validated target primer sets, RT² lncRNA qPCR Assays (Qiagen) and miRCURY LNA miRNA PCR Assay (Qiagen) were used for determining lncRNA and miRNA expression levels, respectively. Detailed information on the primer assays is shown in Table 1. Real-time PCR application based on SYBR Green fluorescence was conducted to detect the lncRNA and miRNA levels by using RT² SYBR Green ROX FAST Mastermix (Qiagen). We performed the PCR in two steps: 1) 95°C for 10 min and 2) 40 cycles consisting of 95°C for 15 s and 60°C for 60 s, respectively. The fluorescence intensity was measured to obtain the Ct (threshold cycle) value for all samples at the end of each cycle. Single peaks are observed in the melting curves for all assays, confirming specific amplification. We determined the hsa-miR-128-3p and PVT1 relative expression levels between the cell line A549 and the non-cancer BEAS-2B cell line by $2^{-\Delta\Delta Ct}$ method by normalising to U6 snRNA and GAPDH expressions (15), and all samples were carried out in triplicate, using biological and technical replicates.

In silico Analysis of lncRNA-miRNA Interactions

It is well established that lncRNAs frequently act as ceRNAs or molecular sponges, thereby binding miRNAs and modulating their biological activities (16). To further investigate the mechanism of PVT1 in lung cancer cell lines, we examined whether it affects the expression profile of miR-128. For this purpose, we used the online bioinformatics tool Starbase-ENCORI (https://rnasysu.com/encori/, access date: 18 February 2025) to show the binding probability of PVT1 with miR-128 directly or not.

Table 1. miRNA and lncRNA assay information

lncRNA Name	Gene ID or miRbase Accession Number	GeneGlobe ID
PVT1	5820	LPH17013A
GAPDH	2597	LPH31725A
U6 snRNA	26827	YP02119464
hsa-miR-128-3p	MIMAT0000424	YP00205995

ID: Identification, lncRNA: long non-coding RNA

Statistical analysis

GraphPad Prism (v8.0) was used to perform the statistical evaluation. Fold change values less than one were converted to fold regulation using the following formula: Fold Regulation = (−1 / fold change value). Fold changes values ≥ 2 and ≤ −2 were interpreted as upregulation and downregulation, respectively. Student's t-test was performed to compare the differences among groups, $p<0.05$ regarded as statistically significant.

RESULTS

lncRNA PVT1 may interact with miR-128 based on in silico analysis

We used Starbase-ENCORI as a bioinformatics tool to forecast the binding possibility of PVT1 with miR-128 (Figure 1). Data from Starbase-ENCORI indicate that PVT1 may bind to miR-128 and downregulate its expression. (CLIP Data: in silico prediction, TMDM score: 1.4343). According to the results of the in silico prediction based on the TMDM score, PVT1 has a relatively high binding capacity with hsa-miR-128-3p.

Real-time quantitative PCR (RT-qPCR) experiment for miR-128 and PVT1

We analysed the expression levels of PVT1 and miR-128 to test whether there was an interaction between their expressions in the lung cell lines. We observed the elevated expression of PVT1 in lung cancerous cell A549, while miR-128 expression was not different compared with the non-cancerous cells BEAS-2B. PVT1 expression increased 4.03-fold; this alteration was statistically significant ($p<0.01$). miR-128 expression was downregulated 1.05-fold, but no significant statistically ($p>0.05$). The expression results of miR-128 and PVT1 are indicated in Figures 2 and 3, respectively. Table 2 also shows the fold regulation and p-value.

miRNA	GeneName	GeneType	TargetSite	Alignment	Type	TDMDScore
hsa-miR-128-3p	PVT1	lncRNA	chr8:127855212-127855234[+]	Target: 5' UAGGAGUCCUGCUGUCACUGUGG 3' miRNA : 3' UUUCUCUGG-CCA-AGUGACACU 5'	7mer-m8	1.4343
hsa-miR-128-3p	PVT1	lncRNA	chr8:128099409-128099429[+]	Target: 5' CACCACCCAGCAUCACUGUGG 3' miRNA : 3' UUUCUCUGGCCAAGUGACACU 5'	7mer-m8	0.7701

Figure 1. lncRNA PVT1 can bind directly to hsa-mir-128-3p. Possible binding regions between PVT1 and miR-128 are shown in the Alignment section.

Table 2. Statistical results and fold regulation values

	Average ΔCt A549	Average ΔCt Control (BEAS-2B)	$2^{-\Delta\Delta Ct}$ A549	p value	Standard Deviation	Fold Regulation
hsa-mir-128-3p	2.55	2.48	0.95	>0.05	0.033	-1.05
lncRNA PVT1	6.17	8.18	-2.01	<0.01*	0.012	4.03
U6 snRNA	0.00	0.00	1.00	>0.05	-	-
GAPDH	0.00	0.00	1.00	>0.05	-	-

Student's t-test: p<0.05*, Ct: Cycle threshold, snRNA: small nucleolar RNA, GAPDH, and u6 snRNA: Reference gene

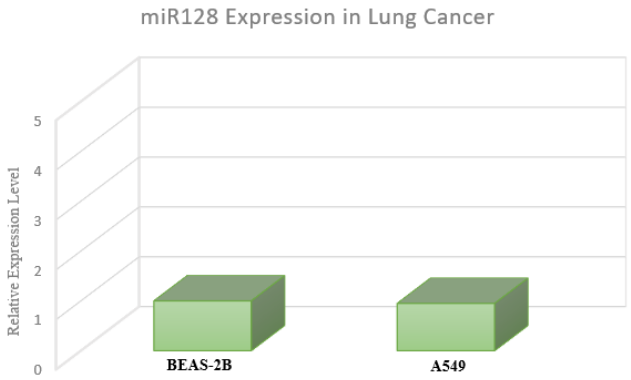

Figure 2. Relative expression level of hsa-miR-128-3p in the lung carcinoma cell line A549. There was no statistically significant difference in the miR-128 expression level between BEAS-2B and A549 cells (p>0.05). Lung cells were cultivated for 48 h before the qRT–PCR experiment, which was performed to determine the fold change.

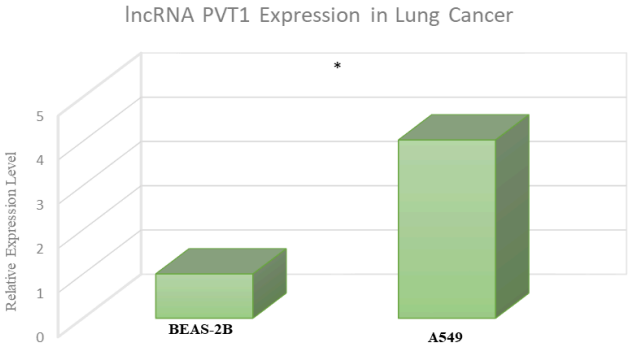

Figure 3. Relative expression level of PVT1 in the lung carcinoma cell line A549. The elevated PVT1 expression level was statistically significant (p<0.01) according to the fold change results obtained from qRT–PCR experiment after 48 h of lung cell culture

DISCUSSION

miRNAs and lncRNAs contribute significantly to the modulation of various biological processes and are often linked to human disorders, including cancer. Understanding the interactions between these two classes of non-coding RNAs is crucial for unravelling the full complexity of gene regulatory networks and enlightening their potential effect for diagnosis as a marker in terms of disease pathophysiology.

The dysfunction of miRNAs has been demonstrated in malignant disorders like cancer via several mechanisms, including the overexpression or downregulation of miRNA genes, improper regulation of miRNAs transcriptionally, and disruptions in the biosynthesis mechanism of miRNA. miR-128 is known as a significant tumour suppressor that inhibits cell migration, tumour growth, and metastasis through its upregulation in cancer cells (17). Numerous studies have demonstrated that miR-128 regulates the progression of cancer by upregulating or downregulating. A recent study showed that the levels of miR-128 are higher in the benign type than in the invasive one for prostate cells (18). A further study has demonstrated a tumour-suppressive function for miR-128-3p in hepatocellular carcinoma (HCC) development and progression (19). miR-128 has been demonstrated to inhibit cell growth and trigger apoptosis in gastric cancer cells (20). miR-128-3p, in a cellular model, has been studied for its ability to decrease cell viability and suppress the ability to form colonies by inducing apoptosis (21). It has been demonstrated that decreased miR-128-3p expression in tissue samples from patients with lung cancer is linked to TNM stage and tumour size (22). Nonetheless, in this study, we showed that the difference in the level of mir-128 expression between the lung carcinoma cells A549 and the non-cancerous cells

BEAS-2B was not statistically significant. Despite the proposal of miR-128 as a broadly applicable cancer-related target, our findings indicate that it does not function as a central mediator in the development of LC, thereby invalidating its use as a therapeutic strategy in LC.

Several cancers have been correlated with aberrant PVT1 expression. Recent studies showed upregulated PVT1 expression in multiple types of cancers in cancer tissues and cell lines, including NSCLC, oral squamous cell carcinoma, gallbladder cancer, pancreatic carcinoma, glioma, and oesophageal cancer. Furthermore, its elevated expression was related to the undesired clinical features of the tumours, such as poor prognosis, invasion, metastasis, higher grade, increased migration and proliferation of cells, and tumour progression (23-29). Studies have shown that lncRNA PVT1 affects tumour progression by inhibiting miRNA expression. PVT1 elimination improved the radiosensitivity through its sponging effect on miR-195 in non-small cell LC (28). PVT1 may facilitate the cell migration and growth in pancreatic carcinoma by sponging miR-488. (29). PVT1 also promotes tumour progression by acting as a sponge endogenously for miR-128 in glioma (12). miR-128 is an essential target of PVT1 in cancer. It was shown that PVT1 contributes to the cell proliferation in breast cancer and epithelial-mesenchymal transition through its binding to miR-128 and FOXQ1 (13). EMT progression is a key factor for the migration of NSCLC cells. Because many human malignancies stem from epithelial tissues, a deeper understanding of the EMT processes could contribute significantly to our knowledge of solid tumour development. Recent studies have identified PVT1 as a key regulator of EMT progression, unveiling a previously unrecognised mechanism underlying this process (29). Downregulation of PVT1 increased the miR-128 level and reduced proliferation in oesophageal squamous cell carcinoma. However, it has been demonstrated that reducing the miR-128 expression level while simultaneously knocking down PVT1 reverses the reduced proliferation, invasion, and migration ability (30). These results emphasise the connection between lncRNA PVT1 and miR-128, operating through various cellular mechanisms linked to EMT and proliferation. Based on the findings, it can be concluded that lncRNA PVT1 and miR-128 bind to their respective target mRNAs competitively; thus, malignancy may develop in cases where PVT1 predominates in the cell due to hampering miR-128 tumour suppressive activity. In our study, we found elevated PVT1 expression in A549 compared with BEAS-2B. However, this increased expression was not accompanied by a decrease in miR-128 expression. This discordance may show that PVT1 does not tend to bind with miR-128 directly, especially in the A549 cell line. As a limitation of our study, we did not use alternative lung cancer cell lines and did not perform induction or inhibition experiments. Further investigations with different cell lines and induction/inhibition experiments should be performed to confirm our findings.

We employed the bioinformatics tool Starbase to support our hypothesis that the expression of PVT1 may inhibit miR-128 expression in LC cells. It has been established that PVT1 has two target sites to bind to miR-128. However, only one site had a high TMDM score, which indicated a higher possibility of interaction between the miRNA and lncRNA. Unlike in silico prediction, elevated PVT1 expression did not directly affect miR-128 expression. We have concluded that PVT1 may act as an endogenous RNA that binds to miR-128 targets competitively. We need to investigate further how lncRNA PVT1 may function as a sponge for miR-128 by modulating its activity. For instance, whole transcriptome sequencing can be used to analyse the alterations in the mRNA profile following the knockdown of miR-128 or lncRNA PVT1.

In conclusion, we examined whether there is any interplay among lncRNA PVT1 and miR-128 in lung cancer cells compared to non-cancerous cells. Our findings reveal that PVT1 expression is elevated in lung cancer cells, while the expression level of miR-128 remains unchanged. This research adds valuable insights to the literature by elucidating the etiopathogenesis of lung cancer through the interaction of PVT1 and miR-128. However, additional analyses are needed to assess the mechanistic interaction between PVT1 and miR-128.

Ethics Committee Approval: For this type of study, there is no need for written informed consent and formal ethics approval.

Informed Consent: For this type of study, there is no need for written informed consent and formal ethics approval.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.N.Y.T., G.N.Ö., R.O.A., E.U., S.T.K., F.H.K.; Data Acquisition- E.N.Y.T., G.N.Ö., R.O.A., E.U., S.T.K.; Data Analysis/Interpretation- E.N.Y.T., F.H.K.; Drafting Manuscript- E.N.Y.T., F.H.K.; Critical Revision of Manuscript- E.N.Y.T., G.N.Ö., R.O.A., E.U., S.T.K., F.H.K.; Final Approval and Accountability- E.N.Y.T., G.N.Ö., R.O.A., E.U., S.T.K., F.H.K.

Conflict of Interest: The authors declare that there is no conflict of interest.

Financial Disclosure The authors declared that this study has received no financial support.

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