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Exploring miR-30b Methylation and MALAT-1 Expression as Diagnostic Biomarkers for Non-Small Cell Lung Cancer

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Abstract

Background: Aberrant methylation and expression of various noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), confer a great potential as tumor markers. This study aimed to investigate miR-30b DNA methylation and Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT-1) expression patterns as potential diagnostic biomarkers for non-small cell lung cancer (NSCLC).

Method: In this cross-sectional study, miR-30b DNA methylation and MALAT-1 expression patterns were first explored using microarray data retrieved from the NSCLC dataset in the Cancer Genome Atlas (TCGA)-LUNG. Then, the obtained results were further validated in internal samples. Subsequently, genomic DNA was extracted and modified by sodium bisulfite to determine DNA methylation using q-MSP. Total RNA was extracted and transcribed to cDNA to measure transcription level by qRT-PCR. GraphPad 6 Prism v.8 was used to perform the statistical analyses. Comparisons between groups in internal samples were conducted by paired student's t-test, while Mann-Whitney U test was used to analyze TCGA-LUNG data ($P < 0.05$).

Results: Our results indicated miR-30b hypermethylation, miR-30b downregulation and lncRNA MALAT-1 overexpression in NSCLC tumor samples compared with marginal normal samples. These changes were significantly associated with the stage of malignancy like lymph node metastasis. Also, using receiver operating characteristic curve analysis, MALAT-1 expression, and miR-30b methylation and expression patterns were found as possible diagnostic biomarkers for NSCLC (Area under the curve was 0.70, 0.67, and 0.74, respectively).

Conclusion: We found involvement of miR-30b hypermethylation and downregulation as well as lncRNA MALAT-1 overexpression with tumor outcomes of NSCLC patients.

Keywords: miR-30b; lncRNA MALT-1; DNA Methylation; Carcinoma, Non-small-cell lung, Neoplasms

Introduction

Lung cancer, the leading cause of cancer-related mortality, encompasses two main histopathological categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).[1,2] NSCLC, accounting for 75% of lung cancer cases, often necessitates primary tumor resection in early stages. However, the 5-year overall survival rate for NSCLC patients remains low at around 15%,[3,4] primarily due to rapid disease progression, late-stage detection, and treatment resistance. While various driver genes have been implicated in NSCLC pathogenesis,[5,6] the molecular mechanisms underlying lung tumorigenesis are not fully elucidated. Therefore, further exploration of molecular changes in NSCLC progression is essential to identify novel therapeutic targets for early diagnosis and treatment.[7,8]

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), an important gene in tumorigenesis, is a long non-coding RNA (lncRNA) normally expressed in various human tissues.[9] Dysregulation of MALAT-1 contributes to the progression of human malignancies, including NSCLC,[10] affecting tumor cell proliferation, apoptosis, epithelial-mesenchymal transition (EMT), invasion, metastasis, and drug resistance, which are all correlated with poor patient prognosis. Studies have shown elevated MALAT-1 expression in various solid tumors, including lung adenocarcinoma,[9,11] breast,[12] gastric,[13] bladder,[14] and pancreatic[15] cancers. MALAT-1 also functions as a competitive endogenous RNA (ceRNA), sequestering and silencing tumor suppressor miRNAs by binding to RNA response elements.[16-18] Consequently, the relationship between MALAT-1 expression and NSCLC prognosis remains a subject of debate.[19]

MicroRNAs (miRNAs), small non-coding RNAs (18-25 nucleotides), play a pivotal role in gene regulation by directly binding to the 3'-untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translation inhibition. MiRNAs are extensively involved in the initiation and progression of various human malignancies, including lung adenocarcinoma,[20] presenting promising therapeutic targets for lung cancer. For instance, the downregulation of miR-340-5p during NSCLC progression has been linked to increased cell proliferation and invasion, while its exogenous overexpression suppressed these activities.[21] MiR-30b, a key member of the miR-30 family, has been implicated in the development of several human cancers, such as lung,[22] glioma,[23] and breast[24] cancers. Previous reports have demonstrated that miR-30b and miR-30c are co-regulated by epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR), functioning as oncogenes by repressing apoptotic regulating molecules like Apoptotic peptidase activating factor 1 (APAF-1) and Bcl-2-like protein 11 (BIM).[25] However, conflicting reports suggest that miR-30b can also act as a tumor suppressor.[26]

Given the gaps in our understanding of the molecular mechanisms driving lung tumorigenesis and the urgent need for novel therapeutic and diagnostic targets, further exploration of key molecular players like MALAT-1 and miR-30b in NSCLC is imperative. Despite advances in identifying driver genes associated with NSCLC, intricate interactions and regulatory networks

involving non-coding RNAs like MALAT-1 and miR-30b remain poorly understood. Therefore, this study aimed to investigate the dysregulation of MALAT-1 and miR-30b in NSCLC, with a focus on elucidating their diagnostic potential and shedding light on their roles in disease progression. Our goal is to fill in these critical gaps in order to provide valuable insights that may lead to more effective early diagnosis and targeted therapies in NSCLC.

Material and Method

In-silico analysis using lung cancer dataset (TCGA-LUNG)

In the first step, the expression levels of MALAT-1 and miR-30b and miR-30b methylation status from available high-throughput experiments for NSCLC were bio-informatically analyzed. We used the available data from The Cancer Genome Atlas (TCGA), which is a public-funded project that presents a comprehensive "atlas" of human cancers' genomic profiles from large cohorts.[27] Then, using Xena Functional Genomics Explorer (https://xena.ucsc.edu/), MALAT-1 and miR-30b expression data were first retrieved from TCGA lung cancer (TCGA-LUNG) dataset and analyzed to pre-evaluate its status in lung cancer patients compared with healthy cases. Besides, methylation levels of miR-30b were analyzed according to beta values of methylation specific probes overlapping with CpG regions in miR-30b promoter obtained from TCGA-LUNG dataset.

Preparation of patient samples

This was a cross-sectional study in Imam Reza Hospital during 2019 to 2021, during which 50 tumor samples and tumor margins were collected as control from eligible patients. The inclusion criteria for NSCL patients in this study were: no chronic diseases, not taking long-term medication, and signing a written consent form. All participants in this study were from Azerbaijanian population living in the northwest of Iran. The participants with hemoptysis, prior radiotherapy or chemotherapy, tuberculosis, or patients who refused to participate in this study were excluded. After obtaining a written informed consent from all participants, lung tissues were collected by bronchoscopy and Needle biopsy techniques as the routine parts of the patient diagnostic approach. The tissue samples were preserved in liquid nitrogen before they were subjected to genomic DNA and RNA extraction. Clinicopathological features of patients with lung cancer are shown at table 1. The present study was approved by the Ethical Committee of Tabriz University of Medical Sciences (approval code: IR.TBZMED.REC.1400.573).

Extraction of genomic DNA and total RNA

The AllPrep DNA/RNA/Protein kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA and RNA according to instructions. Briefly, after smashing using mortar and pestle in liquid nitrogen, the tissue samples were transferred immediately into lysis buffer provided by the kit. In lysis buffer, the samples were homogenized using a needle and syringe and then subjected to DNA and RNA isolation by silica DNA and RNA spin columns. Then, the quality and concentration of extracted nucleic acids were evaluated according to optical density 260 nm and 280 nm using the ThermoFisher's NanoDrop spectrophotometer (Scientific Life Sciences, USA).

The synthesis of cDNA and real-time PCR

Using the BioFACT cDNA synthesis kit (BioFACT, Korea), the extracted total RNA, in amount of 1000 nanogram, was used to synthetizes cDNA according to relevant protocols. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal controls for normalizing transcript levels of MALAT-1 and miR-30b,[28] respectively. Primer sequences are presented in table 1.

Bisulfite conversion and quantitative methylation specific PCR (qMSP)

Briefly, to evaluate methylation status of miR-30b, the extracted genomic DNA, in amount of 1000 nanogram, was treated with bisulfite using EpiTect Bisulfite kit (Qiagen, Hilden, Germany), then purified and recycled according to provided protocols. Bisulfite treatment converts unmethylated cytosine to uracil except for 5-methylcytosines. To perform qMSP test, the methylation-specific primers (Table 1) for miR-30b CpG islands were designed using MethPrimer online program (http://www.urogene.org/methprimer/). Subsequently, BioFACT™ 2X Real-Time PCR Master Mix (BioFACT, Korea) was employed to carry out qMSP in the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) according the following amplification conditions: initial denaturation for 10 min at 95 ℃, 45 cycles of 10 sec denaturation at 95 ℃, 30 sec of annealing at 60 ℃, and 20 sec of extension at 72 ℃; as the final step, a melting curve analysis was evaluated. The reactions were duplicated in a total volume of 20 µl.

Receiver operating characteristic (ROC) curve analysis

The ROC curve analysis was performed to investigate whether MALAT-1 expression and miR-30b methylation patterns possessed a potential as the diagnostic biomarkers through assessing the ability of these molecules to discriminate between groups. Then, the expression and methylation values for NSCLC tumor samples and normal marginal lung tissue samples were considered as patient and control values. Then, GraphPad 6 Prism software was employed to perform ROC curve analysis to evaluate the area under curve (AUC) at confidence interval (CI) of 95%.

Statistical analysis

Data were presented as the mean ± standard error of the experiments and GraphPad 6 Prism v.8 (GraphPad Software, San Diego, CA) was used to carry out the statistical analyses. The Livak method (comparative $2^{-\Delta\Delta CT}$) was employed to evaluate relative MALAT-1 expression and miR-30b methylation levels. For comparison between the groups in internal samples, Paired Student's t-test, independent sample t-test, or ANOVA test was used, and Mann-Whitney U test was used to analyze TCGA-LUNG data. Pearson's correlation test was implemented to assess the potential correlation between scale variables. *P* values less than 0.05 were considered to be statistically significant.

Results

miR-30b hypermethylation and downregulation

MiR-30b methylation levels were first evaluated in NSCLC samples using TCGA-LUNG dataset, which illustrated that miR-30b is significantly hypermethylated ($P < 0.0003$) in NSCLC samples (TCGA-LUNG) compared with control samples (Figure 1A). In consistence with this finding, qMSP results on our internal samples also revealed that the methylation level of miR-30b was significantly higher in NSCLC tumor tissue samples compared with the marginal normal samples ($P = 0.042$; Figure 1B). In addition, we evaluated the expression level of miR-30b in our samples which revealed a significant downregulation of it in NSCLC tumor samples ($P = 0.0002$; Figure 1C). Nonetheless, there was not any data about miR-30b expression level in TCGA-LUNG database.

MALAT-1 overexpression in NSCLC samples

Initial analysis of MALAT-1 expression using TCGA-LUNG dataset evidenced that MALAT-1 had significantly ($P < 0.0001$) higher expression levels in NSCLC samples in comparison with normal lung tissue specimens (Figure 2A). This finding was

further confirmed in our internal samples. The results obtained from qRT-PCR results indicated that this lncRNA was significantly (P = 0.0007) overexpressed in NSCLC tumor samples compared with marginal tissues (Figure 2B).

Correlation/association analysis

The analysis on internal samples divulged a negative correlation between miR-30b expression and its methylation (r =- 0.39 and P = 0.01; Figure 3A) as well as MALAT-1 expression (r =- 0.35 and P = 0.024; Figure 3B). Furthermore, the analysis (Table 2) showed that miR-30b expression was significantly lower in the tumor samples from participants with stage IV compared with those with stage II and stage III (P = 0.003). Tumor samples from patients with lymph node metastasis had significantly lower miR-30b expression compared with those without lymph node metastasis (P = 0.031). Methylation level of miR-30b was significantly higher in the tumor samples from subjects with stage IV compared with those with stage II and stage III (P = 0.034). Tumor samples from patients with lymph node metastasis had significantly higher miR-30b methylation level compared with those without lymph node metastasis (P = 0.0054). Analysis revealed that MALAT-1 expression was significantly higher in the tumor samples from subjects with stage IV compared with those with stage II and stage III (P = 0.022). Tumor samples from patients with lymph node metastasis had significantly higher MALAT-1 expression compared with those without lymph node metastasis (P = 0.0012).

The diagnostic value of MALAT-1 expression and miR-30b methylation in NSCLC

To examine whether MALAT-1 expression and miR-30b methylation and expression status exhibit diagnostic potential for NSCLC, we performed ROC curve analysis on internal samples. The obtained results indicated that AUC for miR-30b expression was 0.74 (P = 0.00014; Figure 4A), for miR-30b methylation was 0.67 (P = 0.0088; Figure 4B), and for MALAT-1 expression was 0.70 (P = 0.001; Figure 4C).

Discussion

This study revealed significant molecular alterations in NSCLC tumor tissues compared with normal marginal samples. Specifically, we observed hypermethylation of miR-30b, which was accompanied by downregulation of this microRNA. Additionally, we found that the lncRNA MALAT-1 was markedly overexpressed in NSCLC samples. These changes in miR-30b and MALAT-1 expression were not only associated with the presence of cancer but were also associated with the stage of malignancy and lymph node metastasis, suggesting their potential as prognostic indicators. Furthermore, our investigation identified these molecular alterations as promising diagnostic biomarkers for NSCLC. Using ROC curve analysis, we determined that MALAT-1 expression and the patterns of miR-30b methylation and expression could effectively distinguish NSCLC from normal tissue. These findings underscore the diagnostic use of these biomarkers in identifying NSCLC and highlight their potential for clinical application in cancer diagnostics and patient management.

Our experiments revealed the miR-30b was significantly downregulated in the NSCLC tumor tissues compared with normal marginal samples. MiR-30 family exhibits downregulation in pancreatic cancer tissues compared with normal pancreatic tissues. Moreover, it was evidenced that the upregulation of miR-30 family could hinder in vivo and in vitro tumorigenesis of pancreatic cancer cells through modulating of cell growth, invasion and migration.[29] Also, Qiu et al. revealed lower expression of miR-

30b-5p in lung tumor cells and tissues and the upregulation of the miR-30b-5p induced apoptosis and prevented A549 and NCI-H1299 cells growth.[26] Moreover, Gu et al. found that miR-30b and miR-30c were both significantly downregulated in cancer tissues compared with normal tissues.[30] On the hand, Qi et al. found that miR-30b was downregulated in NSCLC tumor tissues which led to suppression of metastasis, invasion, proliferation and promoted apoptosis and enhanced sensitivity of the NSCLC cells to EGFR tyrosine kinase inhibitors (EGFR-TKIs) by targeting EGFR.[31] Overall, the collective findings from various studies consistently support the concept that miR-30b downregulation is linked to oncogenic behaviors in diverse tumor samples, as we have also observed in our study. This downregulation of miR-30b is often linked to aggressive tumor characteristics and poor prognosis. However, conflicting results also exist, suggesting that in some contexts, the downregulation of miR-30b may act as a tumor suppressor. The conflicting findings regarding the role of miR-30b in tumors can be attributed to different downstream mechanisms targeted by miR-30b in different tumor types.[32] MiR-30b exerts its effects by regulating the expression of specific target genes involved in various cellular processes such as proliferation, apoptosis, and metastasis. Depending on the cellular context and the specific target genes involved, miR-30b may exhibit either oncogenic or tumor-suppressive functions. For example, in certain tumor types, downregulation of miR-30b may promote tumor growth and metastasis by derepressing oncogenic pathways or by inhibiting tumor suppressor pathways. Conversely, in other tumor contexts, miR-30b downregulation might lead to enhanced tumor suppression by allowing the expression of genes that inhibit tumor growth and metastasis.[32] These insights highlight the complexity of miRNA regulation in cancer and underscore the importance of considering tumor-specific mechanisms when interpreting the role of miR-30b in tumorigenesis.

MiR-30b methylation level was first evaluated in NSCLC samples using TCGA-LUNG dataset, which indicated that miR-30b is significantly hypermethylated in NSCLC samples (TCGA-LUNG) compared with control samples. Results on our internal samples also revealed that the methylation level of miR-30b was significantly higher in NSCLC tumor tissue samples compared with the marginal normal samples. In MIA PaCa-2 Pancreatic cancer cells, hypermethylation of miR-30 was associated with its downregulation. In addition, demethylation agent 5-Aza-dC treatment caused upregulation of the miR-30 family, suggesting the role of miR-30 methylation in regulation of its expression.[29] In gastric tumor cells, it was revealed that the level of miR-30b-5p might be restored through DNA demethylation as well as DNMT1 stimulated miR-30b-5p promoter methylation.[33] The observed hypermethylation of miR-30b represents a significant mechanism underlying the suppression of its expression (as we also indicated negative correlation of miR-30b hypermethylation and its downregulation), which in turn influences tumor behaviors as miR-30 family was associated with the inhibition of EMT, reduced migratory and invasive potentials, and suppression of *in vivo* tumor growth.[34] In various cancers, including NSCLC, pancreatic cancer, and gastric cancer, hypermethylation of miR-30b leads to reduced expression levels of this miRNA. This downregulation is associated with tumor-promoting or tumor-suppressing effects, depending on the specific tumor context and underlying molecular pathways affected by miR-30b dysregulation. Targeting miR-30b methylation as a

therapeutic strategy holds promise for restoring miR-30b expression levels and potentially reversing the oncogenic or tumor-suppressing traits associated with its dysregulation. For instance, the use of demethylation agents like 5-Aza-dC has been shown to restore miR-30b expression in pancreatic and gastric tumor cells. This approach highlights the therapeutic potential of modulating DNA methylation to manipulate miR-30b expression and alter tumor behaviors. It is important to recognize that the functional role of miR-30b—whether it acts as an oncogene or tumor suppressor—varies depending on the specific tumor type and its molecular context. Therefore, when considering therapeutic strategies targeting miR-30b methylation, it is crucial to take into account the tumor-specific characteristics and the underlying mechanisms driving miR-30b dysregulation. This personalized approach will be essential for optimizing the efficacy of miR-30b-targeted therapies in cancer treatment.

Other than regulation through methylation, we also identified potential involvement of lncRNA MALAT-1 in regulation of miR-30b expression. LncRNA MALAT1 was first identified in NSCLC patients that was upregulated in tumors with a raised metastatic predisposition.[35] However, MALAT-1 was later found to be correlated with progression of a wide array of human cancers.[36] Xi et al. indicated that upstream regulator of miR-30b, lncRNA MALAT1, stimulate cisplatin resistance and autophagy in the gastric tumor cell line by suppressing the miR-30b/ autophagy-related protein ATG5 axis which may have a tumor suppressor function in gastric malignancy.[37] Aberrant expression of MALAT-1 was shown to be correlated with NSCLC metastasis, development, and malignancy progression.[38] Besides, MALAT-1 has been also reported to be dysregulated in virtually all types of human malignances and to exhibit a significant association with poor outcomes of patients.[39] Additionally, propofol was shown to promote cisplatin sensitivity by inhibiting autophagy in gastric cancer through MALAT-1/miR-30e/ATG5 axis, implying that MALAT1 stimulated autophagy-associated chemoresistance of gastric cancer cells to cisplatin.[40] Our findings indicate a significant upregulation of the lncRNA MALAT-1 in NSCLC tumors compared with normal tissues, as evidenced by our analysis of the TCGA-LUNG dataset and our internal NSCLC samples. Additionally, we observed a negative correlation between MALAT-1 overexpression and the downregulation of miR-30b. These results suggest that the overexpression of lncRNA MALAT-1 and the hypermethylation of miR-30b are concurrently involved in the regulatory mechanism of miR-30b expression in NSCLC. This implies a potential regulatory relationship between MALAT-1 and miR-30b, where MALAT-1 overexpression may contribute to the downregulation of miR-30b through mechanisms such as epigenetic modifications like methylation.

Downregulation and hypermethylation of miR-30b as well as MALAT-1 overexpression were found to be associated with clinicopathological characteristics of NSCLC subjects. Qiu et al. revealed low expression of the miR-30b-5p in lung tumor cells and tissues associated with poor prognosis and malignant clinical process.[26] In Pancreatic ductal adenocarcinoma (PDAC), downregulation of miR-30 was associated with upregulation of Exportin 1 (XPO1).[29] Upregulation of XPO1 in cancer cells was correlated with the aggressive progression and poor prognosis of cancers such as pancreatic cancer.[41,42] In human lung adenocarcinoma (LAC) tissues, it was illustrated that MALAT-1 overexpression was negatively correlated with miR-429, which was linked to tumor stage, lymph node

metastasis, and tumor size in patients.[42] Our experiments also demonstrated that the downregulation and hypermethylation of miR-30b as well as MALAT-1 overexpression were associated with the stage of malignancy and lymph node metastasis in NSCLC patients. However, we were unable to identify the downstream targets of miR-30b, which limited our ability to gain molecular insights into the precise mechanistic involvement of miR-30b in promoting the cancerous phenotype of NSCLC patients.

In our study, we explored the diagnostic potential of miR-30b methylation and expression levels, along with MALAT-1 expression, for NSCLC. Our findings from ROC curve analysis demonstrated that the upregulation of miR-30b and MALAT-1 expression, as well as the hypermethylation of miR-30b, showed promising diagnostic capability in distinguishing NSCLC tumors from normal samples. These results suggest that the methylation and expression status of miR-30b, along with MALAT-1 expression, could serve as valuable diagnostic biomarkers for NSCLC, potentially facilitating earlier detection and improved management of this disease. Further validation studies are warranted to confirm these findings and assess their clinical utility in NSCLC diagnosis and patient management.

While we made concerted efforts to conduct well-designed experiments to yield robust results, it is important to acknowledge the limitations and caveats of the present study. Firstly, we did not investigate the downstream targets of miR-30b in our samples using approaches such as the dual luciferase assay. Secondly, we did not perform confirmatory mechanistic assays, such as mimicking overexpression of miR-30b in lung cancer cell lines, to elucidate the role of miR-30b in promoting tumor behaviors. Thirdly, we did not use demethylation agents to explore the relationship between altered methylation of miR-30b and its expression levels. These limitations highlight areas for further investigation to enhance our understanding of the molecular mechanisms underlying miR-30b dysregulation in lung cancer.

Conclusion

The findings of our study confirm and expand upon existing knowledge regarding the molecular characteristics of NSCLC. Our research demonstrated that MALAT-1 overexpression, along with miR-30b hypermethylation and downregulation, are concurrent events in lung tumorigenesis. Notably, these molecular changes were found to correlate with advanced clinical stages and the presence of lymph node metastasis in NSCLC patients. Moreover, our study highlights the potential of MALAT-1 and miR-30b as promising diagnostic markers for NSCLC, offering valuable insights into their clinical relevance. We also observed a significant correlation between MALAT-1 expression and miR-30b methylation and expression patterns, suggesting a potential interplay between these molecular alterations in lung cancer initiation and progression. While our findings provide important implications for NSCLC diagnosis and understanding of its underlying mechanisms, further validation studies are warranted to elucidate the precise roles and interactions of MALAT-1 and miR-30b in lung cancer pathogenesis. Future research should focus on unraveling the mechanistic underpinnings of these molecular changes and exploring their therapeutic implications for improving patient outcomes in NSCLC.

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Data Availability

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

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Conflict of Interest

None declared.

Authors' Contribution

M.M.: Conceptualization, Study design, Writing - Original Draft; **A.S:** Study design, Writing - Original Draft, conducted literature searches and selected relevant articles; **H.Z:** Study design, Conducted the literature search, selected relevant articles, and critically analyzed the information and reviewed the final draft; **M.A:** Study design, Data gathering, Drafted specific sections of the manuscript; **Sh.H:** Study design, data gathering, sample collection, methodology; **V.Z:** Study design, Data gathering, Analyzed and synthesized information from selected articles; **H.S.J:** Study design, drafting and reviewing the manuscript, approved the final version of the manuscript; **M.R:** Study design, Project administration, Conceptualization and design of the work, writing review and editing; All authors have contributed to the conception or design of the work or the data acquisition and analysis, or interpretation of data for the work. Also, all authors read or review and approved the final manuscript and agree to be 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.

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Table 1. Sequence of primers used for transcript and methylation measurements

Gene	Primer sequence	Annealing temp (°C)
MALAT-1	F: 5′-GAATTGCGTCATTTAAAGCCTAGTT-3′ R: 5′-GTTTCATCCTACCACTCCCAATTAAT-3′	59
GAPDH	F: 5′-CAAGATCATCAGCAATGCCTCC-3′ R: 5′-GCCATCACGCCACAGTTTCC-3′	59
miR-30b methylation	F: 5′-CAAGATTGTAAACATCCTA-3′ R:5′-CCAGTGCAGGGTCCGAGGTA-3′	60
miR-30b expression	miR-30bstemloop: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACAGGATGTTT 5′-CCAGTGCAGGGTCCGAGGTA-3′ 5′-CGTAGACGTGTAAACATCCT-3′	60
U6	U6-stem-loop GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACAAAAATAT U6-forward GCTTCGGCAGCACATATACTAAAAT 59 U6-reverse CGCTTCACGAATTTGCGTGTCAT	60

MALAT-1; Metastasis associated lung adenocarcinoma transcript 1; GAPDH; Glyceraldehyde-3-phosphate dehydrogenase

Table 2. Association of transcript and methylation of genes and clinicopathological features of patients.

Feature	Count	miR-30b expression		miR-30b methylation		MALAT-1 expression	
		Value	*P* value	Value	*P* value	Value	*P* value
Age							
<55	23 (%46)	1.13 ± 0.16	0.81	53.36 ± 25	0.87	1.74 ± 0.21	0.290
>55	27 (%46)	1.21 ± 0.18		52.21± 21		1.54±0.19	
Gender							
Male	26 (%52)	1.34 ± 0.15	0.11	54.26 ± 28	0.34	1.67 ± 0.14	0.350
Female	24 (%48)	1.29 ± 0.24		53.31 ± 21		1.84 ± 0.19	
Stage of malignancy							
Stage II	23 (%46)	1.09 ± 0.07	0.003	53.7 ± 14	0.034	1.64 ± 0.09	0.022
Stage III	25 (%50)	1.25 ±0.11		59.3 ± 20		1.79 ± 0.17	
Stage IV	2 (%4)	1.49 ± 0.05		72.5 ± 5.1		1.83 ± 0.06	
Lymph node metastasis							
Yes	29 (%58)	1.39 ± 0.18	0.031	67.86 ± 30	0.0054	1.79 ± 0.19	0.0012
No	21 (%42)	1.13 ± 0.17		50.23 ± 21		1.63 ± 0.14	

miR: microRNA; MALAT-1: Metastasis associated lung adenocarcinoma transcript 1

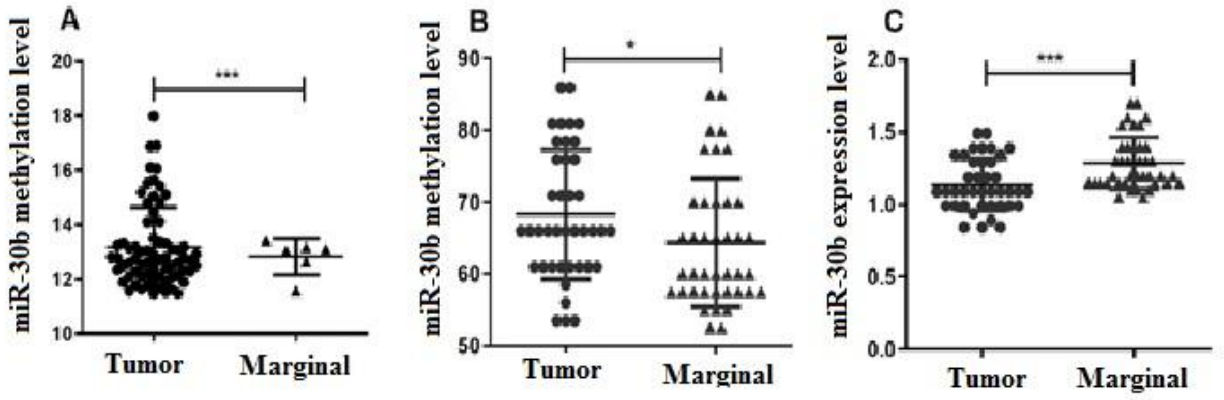

Figure 1. Expression and methylation of miR-30b: Comparison of miR-30b overall methylation levels between NSCLC and normal samples using TCGA-LUNG dataset (A). MiR-30b shows significantly hypermethylated status in NSCLC tumors compared with marginal control. MiR-30b methylation (B) and expression (C) patterns in internal tissue samples measured by q-MSP and qPCR. The obtained results showed that miR-30b was significantly hypermethylated and downregulated in NSCLC tumors in comparison with normal marginal tissues ($^{*}P < 0.05$, $^{***}P < 0.0001$).

miR: microRNA; MALAT-1: Metastasis associated lung adenocarcinoma transcript 1; NSCLC: Non-small cell lung carcinoma; TCGA: The cancer genome atlas; qMSP: Quantitative methylation specific PCR

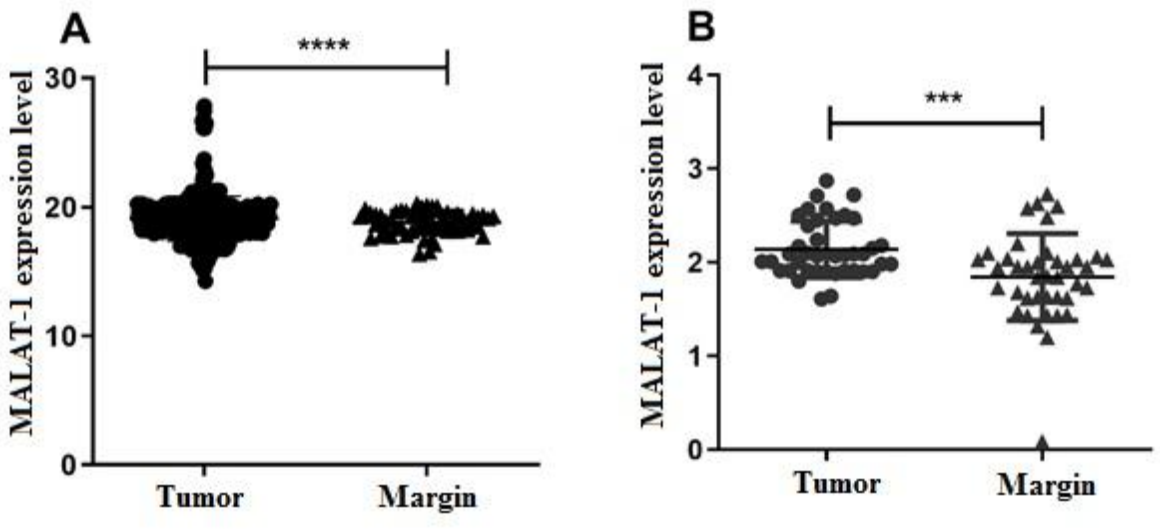

Figure 2. MALAT-1 expression pattern: Comparison of MALAT-1 overall expression levels between NSCLC and normal marginal samples using TCGA-LUNG dataset. MALAT-1 shows significant overexpression in NSCLC tumors compared with marginal samples (A). MALAT-1 expression pattern in internal tumor tissue samples as measured by q-PCR (B). The obtained results showed that MALAT-1 was significantly overexpressed in NSCLC tumor samples in comparison with normal adjacent tissues ($^{***}P < 0.0001$, $^{****}P < 0.00001$).

miR: MicroRNA; MALAT-1: Metastasis associated lung adenocarcinoma transcript 1; NSCLC: Non-small cell lung carcinoma; TCGA: The cancer genome atlas

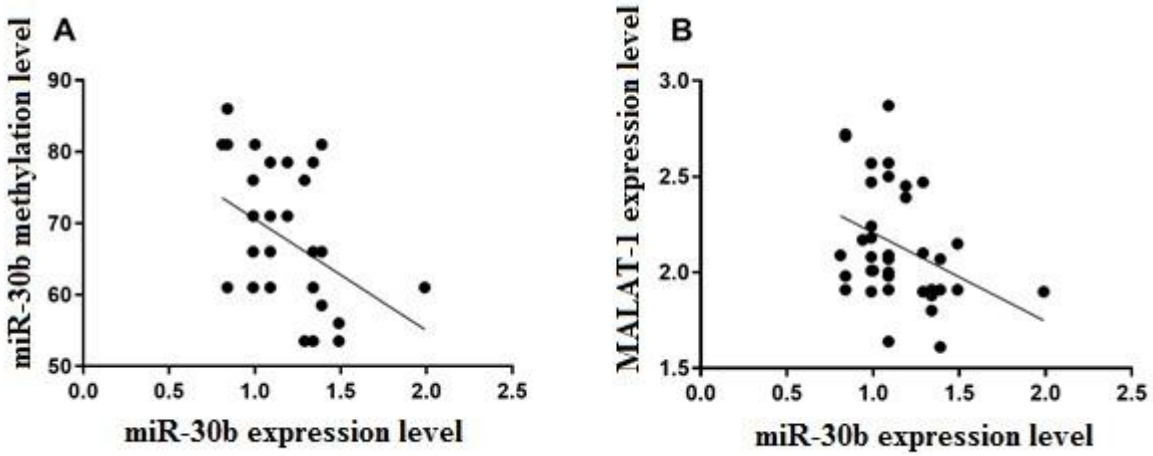

Figure 3. Correlation analysis: MiR-30b expression level was correlated significantly with miR-30b methylation (A) and MALAT-1 expression (B) in the NSCLC internal tumor samples as implemented through Pearson's correlation test.

miR: MicroRNA; MALAT-1: Metastasis associated lung adenocarcinoma transcript 1; NSCLC: Non-small cell lung carcinoma

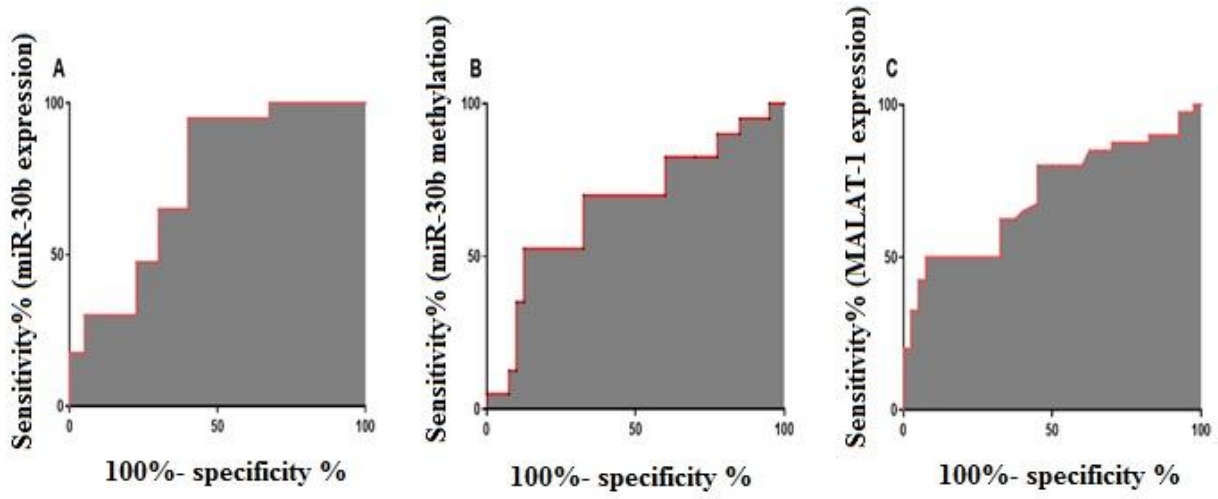

Figure 4. ROC curve analysis: ROC curve analysis was performed on NSCLC internal samples and AUC was calculated for miR-30b expression (A), miR-30b methylation (B), and MALAT-1 expression (C).

miR: MicroRNA; MALAT-1: Metastasis associated lung adenocarcinoma transcript 1; NSCLC: Non-small cell lung carcinoma; ROC: Receiver operating characteristics; AUC: Area under curve