

Original Article

Running Title: FTH-1 and SHOX2 Methylation in Lung Cancer

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Aberrant DNA Methylation of FTH-1 and SHOX2 Contributes to Lung Cancer Progression

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Abstract

Background: Lung cancer is the most lethal malignancy in the world due to its poor prognosis. DNA methylation change has been identified as a valuable target for cancer, diagnosis, and prognosis. Ferritin heavy chain 1 (FTH-1) and SHOX homeobox 2 (SHOX2) DNA methylation were investigated in non-small cell lung cancer (NSCLC) as novel epigenetic biomarkers.

Method: In this case-control study, we initially evaluated the diagnostic value of FTH-1 and SHOX2 DNA methylation, and the Cancer Genome Atlas (TCGA) data on the methylation profile of NSCLC was analyzed. Whole DNA was extracted and bisulfite modification was performed. Then, the methylation status of FTH-1 and SHOX2 was evaluated using Quantitative Methylation Specific polymerase chain reaction (PCR) (qMSP). We used GraphPad Prism version 6.00 program for statistical analysis. Mann-Whitney U test (TCGA-LUNG), paired t-test (internal samples) and receiver operating characteristic (ROC) curve analysis were used to evaluate the statistical differences of DNA methylation between NSCLC tissues samples and adjacent normal specimens ($P < 0.05$, mean \pm SD).

Results: TCGA and q-MSP results showed significant FTH-1 hypomethylation and SHOX2 hypermethylation in NSCLC tissues in comparison with margin specimens. Also, FTH-1 and SHOX2 methylation levels were significantly associated with the clinical stage of malignancy. Furthermore, The ROC curve analysis revealed that the area under the curve (AUC) values for FTH-1 and SHOX2 were determined to be 0.751 and 0.8676, respectively. This indicates the importance of FTH-1 and SHOX2 as diagnostic biomarkers for NSCLC.

Conclusion: This study indicates that FTH-1 and SHOX2 methylation could be promising targets for liquid biopsy application of lung cancer.

Keywords: DNA methylation, Epigenetic, Carcinoma, Non-small-cell lung

Introduction

In 2018, the World Health Organization (WHO) reported that lung cancer killed 2.0 million people. In recent years, efforts have been made to provide early detection of lung cancer by expanding screening in high-risk populations.^{1,2} The epigenetic events (like Abnormal DNA methylation), are a source of biomarkers for the screening, diagnosis, and prognosis of cancers that occur during carcinogenesis.³ Important roles of DNA methylation include regulating gene expression, epigenetic alterations, and maintaining cellular identity, which often occur in tumorigenesis.⁴

Evidence shows that aberrant methylation happens through cancer progression in two ways; global hypomethylation and regional hypermethylation. DNA hypermethylation especially occurs in CpG islands within the promoter region of tumor suppressor genes in malignant cells,⁵ leading to post-transcriptional silencing of associated genes and subsequent tumor progression.⁶ Many studies have shown that DNA methylation abnormalities are also associated with the incidence and development of lung cancer.⁷ The aberrant methylation of certain genes in tumor tissues compared with normal ones suggests that dysregulation of these genes may be involved in tumor progression, invasion, and metastasis by modulating various mechanisms.⁸ Evidence illustrated that hypermethylation within the promoter region of SHOX2 (the short stature homeobox gene 2) is involved in its regulation and could serve as a potential diagnostic biomarker for lung cancer screening at early or advanced stages.^{9,10} Subsequently, SHOX2 methylation has been under consideration in an increasing number of studies investigating its diagnostic value in lung cancer.¹¹

Another gene that has been introduced as a novel biomarker for lung cancer is the FTH1 gene. It was shown that Ferritin-heavy

polypeptide 1 is involved in various cellular processes, including cytokine signaling, adaptive immune system regulation, and cell death.¹² Besides, FTH1 has been illustrated to bind high molecular weight kininogen (HKa) and repress its anti-angiogenic activity on epithelial cells during inflammation and tumor progression in vivo and in vitro.¹³ It was shown that FTH1 is downregulated through tumorigenesis, and functions as a tumor suppressor in non-small cell lung cancer (NSCLC),¹⁴ breast cancer,¹⁵ and ovarian cancer.¹⁶ However, FTH1 also shows oncogenic properties in metastatic melanoma cells.¹⁷

Therefore, given their significance through tumor progression, the present study aimed to evaluate whether SHOX2 and FTH-1 methylation levels could serve as valuable diagnostic targets for lung cancer.

Materials and Methods

Validation of FTH-1 and SHOX methylation in NSCLC samples

Preparation of patient samples

In the present case-control study, tissue samples were collected from 50 patients with lung cancer and 50 healthy people in Imam Reza Hospital for three years, from 2018 to 2021. All patients who participated in the present study were from the north-west population of Iran (Azarbaijan) (Table 1). Patients with radiotherapy or chemotherapy, tuberculosis history, or patients who refused to participate in the study were excluded. An informed written consent was obtained from all participants, and the lung tissues of participants were collected by bronchoscopy and needle biopsy techniques, as the routine parts of the patient diagnostic approach. Tissue samples were maintained in liquid nitrogen till DNA extraction was performed. This study was approved by the Ethical Committee of Tabriz University of Medical Sciences (Ethical Code is IR.TBZMED.REC.1400.575).

In silico investigation of DNA methylation in lung cancer

DNA methylation status of the FTH1 gene was initially analyzed using TCGA methylation datasets on lung cancer (TCGA-LUNG dataset). As a public-funded project, TCGA represents a comprehensive “atlas” of cancer genomic profiles from large cohorts. To evaluate FTH1 methylation status, the beta value for the cg24898753 probe overlapping with CpG islands within FTH1 promoter in lung cancer samples and normal samples was attained from TCGA-LUNG dataset (The Illumina HumanMethylation27 Bead Chip) using Xena Functional Genomics Explorer (<https://xena.ucsc.edu/>). Then, changes in FTH1 DNA methylation were compared between the two groups according to mean values.

DNA Extraction and bisulfite conversion

To extract genomic DNA, tissue samples were mashed using mortar and pestle in liquid nitrogen and then transferred rapidly into extraction lysis buffer. Then, samples were homogenized and DNA isolation was done by DNeasy Blood and Tissue Kit kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After, extracted DNA qualification was determined at 260 nm and 280 nm absorbance wavelengths using the NanoDrop spectrophotometer (ThermoFisher Scientific Life Sciences, USA). Then, bisulfite modification was done to convert unmethylated cytosine to uracil except for the 5-methylcytosines.

Quantitative methylation specific polymerase chain reaction (PCR)

We use the quantitative methylation-specific PCR (q-MSP) to determine the DNA methylation status of FTH-1 and SHOX genes in experimental samples. To do so, we retrieved the sequence of CpG islands within FTH-1 and SHOX promoter from the UCSC genome browser (<https://genome.ucsc.edu/>). Then, using MethPrimer program (<http://www.urogene.org/methprimer/>),

methylation-specific primers were designed, and q-MSP was performed in the Applied Biosystems Step One Plus Real-Time PCR System (USA) using 2X Real-Time PCR Master Mix (BioFACT™, South Korea). The reaction occurred under the following conditions: Pre-heating at 95 °C for 13 min, 45 cycles of denaturation for 10 s at 94 °C, 30 s annealing at 60 °C and 20 s extensions at 72 °C followed by a melting curve analysis. The comparative $2^{-\Delta\Delta C_t}$ method was used for the relative quantification of target gene methylation. The sequences of designed primers are shown in table 2.

Receiver operating characteristic (ROC) curve analysis

We used the ROC curve analysis to evaluate whether FTH-1 and SHOX2 DNA methylation status could be considered as the potential detection biomarkers to effectively discriminate NSCLC patients from healthy cases. Thus, the area under the curve (AUC) in TCGA-LUNG cohort and experimental tissue samples was estimated.

Statistical analysis

GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA) was used for data analysis. In accordance with data distribution, the Mann-Whitney U test (TCGA-LUNG) and paired t-test (internal samples) were used to evaluate the statistical differences of DNA methylation between NSCLC tissue samples and adjacent normal specimens. ROC curve analysis was used to evaluate the probability of each miRNA as a biomarker ($P < 0.05$, mean \pm SD).

Results

FTH-1 hypomethylation in correlated with NSCLC progression

FTH-1 DNA methylation was first investigated in the TCGA-LUNG dataset containing DNA methylation profiles of 277 lung cancer tissue samples and 168 noncancerous lung specimens. The results obtained based on cg24898753 probe

overlapping with FTH-1 promoter illustrated that FTH-1 is significantly ($P < 0.0001$) hypomethylated in NSCLC tissue samples as compared with normal lung tissue specimens (Figure 1). To further validate FTH-1 hypomethylation through lung tumorigenesis, a Q-MSP test was performed on our internal samples, including NSCLC tissue samples and adjacent normal specimens. As shown in figure 2, the results obtained from q-MSP also showed that FTH-1 is significantly ($P = 0.001$) hypomethylated through lung cancer progression (Figure 1). Furthermore, the association between clinic-pathological properties of patients and FTH-1 methylation was investigated in internal samples. It was shown that FTH-1 hypomethylation is significantly correlated with tumor stage ($P = 0.043$) and lymph node metastasis ($P = 0.029$). However, there was no significant relationship between FTH-1 methylation levels and other parameters including age and sex. The data are presented in table 3.

SHOX2 hypermethylation in NSCLC samples

SHOX2 DNA methylation was first investigated in the TCGA-LUNG dataset containing DNA methylation profiles of lung cancer tissue samples and noncancerous lung specimens. The results obtained based on cg17191178 probe overlapping with SHOX2 promoter illustrated that SHOX2 is significantly ($P < 0.0372$) hypermethylated in NSCLC tissue samples compared with normal lung tissue specimens (Figure 2). In our samples, the results from q-MSP indicated that SHOX2 exhibits significantly higher methylation levels in NSCLC samples compared with adjacent healthy tissues, as depicted in Figure 2. Besides, SHOX2 methylation levels was shown to be significantly correlated with tumor stage ($P = 0.031$) and lymph node metastasis ($P = 0.042$) in NSCLN patients. However, there was no significant relationship between

SHOX2 methylation levels and other parameters including age and sex. The data are presented in table 3.

High diagnostic value of FTH-1 and SHOX2 methylation in NSCLC

To further examine whether aberrant methylation of FTH-1 and SHOX2 could be valuable diagnosis biomarkers in NSCLC, ROC curve analysis was performed using q-MSP results. As seen in Figure 3, the results implied that FTH-1 and SHOX2 methylation exhibit high potential as the detection biomarkers (AUC = 0.82, $P < 0.0001$ and AUC = 0.64, $P < 0.024$, respectively) to discriminate NSCLC patients from normal ones.

Discussion

This study evaluated the aberrant DNA methylation of FTH1 and SHOX2 in lung cancer progression. Firstly, FTH1 methylation as a biomarker for NSCLC was investigated using TCGA-LUNG datasets. The results showed that FTH1 is significantly hypomethylated in NSCLC samples compared with normal ones. FTH1 hypomethylation through lung tumorigenesis was further validated in a set of experimental samples, including 50 NSCLC tissue samples and 50 adjacent normal tissue samples, using the q-MSP technique method; showing a significant correlation between FTH1 methylation levels, tumor and lymph node metastasis. Moreover, ROC curve analysis indicates that FTH1 methylation has a biomarker potential in NSCLC patients (AUC = 0.82). Additionally, SHOX2 methylation status was also investigated in internal samples in the present study. Our results established that SHOX2, known as an important tumor suppressor gene, is significantly hypermethylated through NSCLC progression. Also, a significant correlation was found between SHOX2 methylation levels and clinical features, including lymph node metastasis and stage of

malignancy. Also, the diagnostic value of SHOX2 methylation for NSCLC was confirmed by ROC curve analysis, which estimated an AUC value equal to 0.64.

Early diagnosis of lung cancer remains a critical need to improve treatment outcomes. Abnormal methylation has also been shown to be associated with tumor occurrence and development through abnormal gene expression which leads to dysregulation of downstream signaling pathways.¹⁸ Besides, growing studies have established that aberrant DNA methylation of various genes is correlated with tumor aggressiveness and progression,^{19,20} illustrating its significance as a diagnostic and prognostic target.

Also, FTH-1 plays key roles in a wide array of cellular processes, including cytokine signaling, adaptive immunity,²¹ and cell death.¹² FTH-1 has been previously illustrated in triple-negative breast cancer to participate in the activation of IFN α/β and IFN γ signaling.²² Furthermore, its upregulation was shown to affect neural progenitor cell migration, proliferation, and apoptosis, *in vitro*.²³ FTH-1 dysregulation was also found to be involved in the pathogenesis of various human malignancies, including lung, liver, and prostate cancers. Epigenetic silencing of FTH-1 also could inhibit cell growth of Hep-G2 liver cancer cells; illustrating its significance in human cancers.²⁴ Amjad et al. reveal that suppressing of FTH1 induces mammosphere formation and cell proliferation, which leads to high expression of cellular myelocytomatosis (c-MYC), and decreased sensitivity of breast cancer to chemotherapy.²⁵ They also found that the upregulation of FTH1 reduces cell proliferation and growth via suppression of c-MYC oncogene.²⁵ Also, Lu et al. indicated that suppressing of FTH-1 decreases prostate cancer metastasis via the downregulation of S100A4, S100A2, and S100P expression.²⁶ Biamonte et al. revealed that suppression of

FTH1 in K562 cells promotes the expression of some onco-miRNAs, and boosts chemoresistance in K562 and SKOV3 cells.²⁷ Also, Li et al. found that the upregulation of FTH1P3 lncRNA increased the migration and invasiveness of NSCLC via activating epithelial-mesenchymal transition.²⁸ Liu et al. found circulating lncRNA FTH1P2 (exosome RNAs) in lung adenocarcinoma patient's blood which was considerably correlated with the prognosis and diagnosis of NSCLC.²⁹

As a member of the paired homeobox gene family short stature homeobox located at chr3q25.32, SHOX2 has been identified to be highly expressed in various malignancies. However, SHOX2 functions and involved molecular mechanisms through lung tumorigenesis are rarely known. Li et al. established that SHOX2 dysregulation may participate in lung cancer incidence, metastasis, and recurrence.³⁰ Also, SHOX2 methylation levels were shown to be associated with the tumor stage in lung cancer malignancies and were higher in advanced stages of lung cancer patients.³¹ In another study, Zeng et al. found that miR-375 upregulation and SHOX2 hypermethylation led to worse pathological features in NSCLC patients. They also showed that SHOX2 methylation and upregulation of miR-375 together had better diagnostic worth than the single diagnosis.³² Also, Vo et al. indicated that methylation of SHOX2 is the acceptable marker to separate from benign malignant lung tissue in Vietnamese patients.³³ Kneip et al. demonstrated that DNA methylation of SHOX2 could be used as a biomarker to recognize lung cancer patients from healthy groups at a sensitivity and specificity of 60% and 90% respectively.³⁴ Feng et al. detected that 93.3% of NSCLC patients hypermethylated in SHOX2 gene in tumor tissue samples (83 from 89 NSCLC patients).³⁵

Given the limitations of this study, to get more precise outcomes and better understanding, we need further research with a higher number of patients and related genes with FTH-1 and SHOX2 to be used as a prognostic and diagnostic factor in lung cancer.

Conclusion

Given the high mortality rate of lung cancer, mostly due to prognosis, identification and validation of novel diagnostic biomarkers remain the constant need for the better management of this malignancy. Therefore, in the present study, FTH-1 and SHOX2 methylation levels was investigated in lung cancer. Taken together, our results imply that FTH-1 and SHOX2 exhibit aberrant DNA methylation levels through lung tumorigenesis, and they could be considered as valuable targets for the development of novel diagnostic application based on non-invasive methods such as liquid biopsy.

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Authors' Contribution

S.T: Conceptualization, 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:** Data gathering, Drafted specific sections of the manuscript; **Sh.H:** data gathering, Sample Collection, Methodology,; **V.Z:** Data

gathering, Analyzed and synthesized information from selected articles, and participation in drafting; **H.S.J:** study design, drafting and reviewing the manuscript, Approved the final version of the manuscript; **M.R:** Project administration, Conceptualization and design of the work, writing review and editing;

All authors have read 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.

Conflict of Interest

None declared.

References

1. Shanehbandi D, Asadi M, Seyedrezazadeh E, Zafari V, Shekari N, Akbari M, et al. MicroRNA-based biomarkers in lung cancer: recent advances and potential applications. *Curr Mol Med.* 2023;23(7):648-67. doi: 10.2174/2772432817666220520085719.
2. Zarredar H, Farajnia S, Ansarin K, Baradaran B, Aria M, Asadi M. Synergistic effect of novel EGFR inhibitor AZD8931 and p38 α siRNA in lung adenocarcinoma cancer cells. *Anticancer Agents Med Chem.* 2019;19(5):638-44. doi: 10.2174/1871520619666190301125203.
3. Han X, Wang J, Sun Y. Circulating tumor DNA as biomarkers for cancer detection. *Genomics Proteomics Bioinformatics.* 2017;15(2):59-72. doi: 10.1016/j.gpb.2016.12.004.
4. Vizoso M, Puig M, Carmona FJ, Maqueda M, Velásquez A, Gómez A, et al. Aberrant DNA methylation in non-small cell lung cancer-associated

- fibroblasts. *Carcinogenesis*. 2015;36(12):1453-63. doi: 10.1093/carcin/bgv146.
5. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet*. 2012;13(10):679-92. doi: 10.1038/nrg3270.
 6. Ehrlich M. DNA hypermethylation in disease: mechanisms and clinical relevance. *Epigenetics*. 2019;14(12):1141-63. doi: 10.1080/15592294.2019.1638701.
 7. Darwiche K, Zarogoulidis P, Baehner K, Welter S, Tetzner R, Wohlschlaeger J, et al. Assessment of SHOX2 methylation in EBUS-TBNA specimen improves accuracy in lung cancer staging. *Ann Oncol*. 2013;24(11):2866-70. doi: 10.1093/annonc/mdt365.
 8. Peng X, Liu X, Xu L, Li Y, Wang H, Song L, et al. The mSHOX2 is capable of assessing the therapeutic effect and predicting the prognosis of stage IV lung cancer. *J Thorac Dis*. 2019;11(6):2458-69. doi: 10.21037/jtd.2019.05.81.
 9. Dietrich D, Kneip C, Raji O, Liloglou T, Seegebarth A, Schlegel T, et al. Performance evaluation of the DNA methylation biomarker SHOX2 for the aid in diagnosis of lung cancer based on the analysis of bronchial aspirates. *Int J Oncol*. 2012;40(3):825-32. doi: 10.3892/ijo.2011.1264.
 10. Shanebandi D, Saei AA, Zarredar H, Barzegari A. Vibration and glycerol-mediated plasmid DNA transformation for Escherichia coli. *FEMS Microbiol Lett*. 2013;348(1):74-8. doi: 10.1111/1574-6968.12247.
 11. Li N, Zeng Y, Huang J. Signaling pathways and clinical application of RASSF1A and SHOX2 in lung cancer. *J Cancer Res Clin Oncol*. 2020;146(6):1379-93. doi: 10.1007/s00432-020-03188-9.
 12. Liu F, Du ZY, He JL, Liu XQ, Yu QB, Wang YX. FTH1 binds to Daxx and inhibits Daxx-mediated cell apoptosis. *Mol Biol Rep*. 2012;39(2):873-9. doi: 10.1007/s11033-011-0811-5.
 13. Tesfay L, Huhn AJ, Hatcher H, Torti FM, Torti SV. Ferritin blocks inhibitory effects of two-chain high molecular weight kininogen (HKa) on adhesion and survival signaling in endothelial cells. *PLoS One*. 2012;7(7):e40030. doi: 10.1371/journal.pone.0040030.
 14. Biamonte F, Battaglia AM, Zolea F, Oliveira DM, Aversa I, Santamaria G, et al. Ferritin heavy subunit enhances apoptosis of non-small cell lung cancer cells through modulation of miR-125b/p53 axis. *Cell Death Dis*. 2018;9(12):1174. doi: 10.1038/s41419-018-1216-3.
 15. Aversa I, Zolea F, Ieranò C, Bulotta S, Trotta AM, Faniello MC, et al. Epithelial-to-mesenchymal transition in FHC-silenced cells: the role of CXCR4/CXCL12 axis. *J Exp Clin Cancer Res*. 2017;36(1):104. doi: 10.1186/s13046-017-0571-8.
 16. Lobello N, Biamonte F, Pisanu ME, Faniello MC, Jakopin Ž, Chiarella E, et al. Ferritin heavy chain is a negative regulator of ovarian cancer stem cell expansion and epithelial to mesenchymal transition. *Oncotarget*. 2016;7(38):62019-62033. doi: 10.18632/oncotarget.11495.
 17. Yao F, Cui X, Zhang Y, Bei Z, Wang H, Zhao D, et al. Iron regulatory protein 1 promotes ferroptosis by sustaining cellular iron homeostasis in melanoma. *Oncol Lett*.

- 2021;22(3):657. doi: 10.3892/ol.2021.12918.
18. Goldstein M, Kastan MB. The DNA damage response: implications for tumor responses to radiation and chemotherapy. *Annu Rev Med.* 2015;66:129-43. doi: 10.1146/annurev-med-081313-121208.
 19. Cheng J, Wei D, Ji Y, Chen L, Yang L, Li G, et al. Integrative analysis of DNA methylation and gene expression reveals hepatocellular carcinoma-specific diagnostic biomarkers. *Genome Med.* 2018;10(1):42. doi: 10.1186/s13073-018-0548-z.
 20. Yang Z, Qi W, Sun L, Zhou H, Zhou B, Hu Y. DNA methylation analysis of selected genes for the detection of early-stage lung cancer using circulating cell-free DNA. *Adv Clin Exp Med.* 2019;28(3):355-60. doi: 10.17219/acem/84935.
 21. Tang D, Chen X, Kang R, Kroemer G. Ferroptosis: molecular mechanisms and health implications. *Cell Res.* 2021;31(2):107-25. doi: 10.1038/s41422-020-00441-1.
 22. Liu NQ, De Marchi T, Timmermans AM, Beekhof R, Trapman-Jansen AM, Foekens R, et al. Ferritin heavy chain in triple negative breast cancer: a favorable prognostic marker that relates to a cluster of differentiation 8 positive (CD8+) effector T-cell response. *Mol Cell Proteomics.* 2014;13(7):1814-27. doi: 10.1074/mcp.M113.037176.
 23. Feng Y, Liu Q, Zhu J, Xie F, Li L. Efficiency of ferritin as an MRI reporter gene in NPC cells is enhanced by iron supplementation. *J Biomed Biotechnol.* 2012;2012:434878. doi: 10.1155/2012/434878.
 24. Muhammad JS, Bajbouj K, Shafarin J, Hamad M. Estrogen-induced epigenetic silencing of FTH1 and TFRC genes reduces liver cancer cell growth and survival. *Epigenetics.* 2020;15(12):1302-18. doi: 10.1080/15592294.2020.1770917.
 25. Ali A, Shafarin J, Abu Jabal R, Aljabi N, Hamad M, Sualeh Muhammad J, et al. Ferritin heavy chain (FTH1) exerts significant antigrowth effects in breast cancer cells by inhibiting the expression of c-MYC. *FEBS Open Bio.* 2021;11(11):3101-14. doi: 10.1002/2211-5463.13303.
 26. Lu C, Zhao H, Luo C, Lei T, Zhang M. Knockdown of ferritin heavy chain (FTH) inhibits the migration of prostate cancer through reducing S100A4, S100A2, and S100P expression. *Transl Cancer Res.* 2020;9(9):5418-29. doi: 10.21037/tcr-19-2852.
 27. Biamonte F, Zolea F, Bisognin A, Di Sanzo M, Saccoman C, Scumaci D, et al. H-ferritin-regulated microRNAs modulate gene expression in K562 cells. *PLoS One.* 2015;10(3):e0122105. doi: 10.1371/journal.pone.0122105.
 28. Li Z, Wang Y. Long non-coding RNA FTH1P3 promotes the metastasis and aggressiveness of non-small cell lung carcinoma by inducing epithelial-mesenchymal transition. *Int J Clin Exp Pathol.* 2019;12(10):3782-90. PMID: 31933766
 29. Liu XQ, Tufman A, Kiefl R, Li GF, Ma QL, Huber RM. Identification of lung adenocarcinoma-specific exosome RNAs in peripheral blood by RNA-Seq analysis. *Eur Rev Med Pharmacol Sci.* 2020;24(4):1877-86. doi: 10.26355/eurev_202002_20366.

30. Li N, Zeng Y, Tai M, Lin B, Zhu D, Luo Y, et al. Analysis of the prognostic value and gene expression mechanism of SHOX2 in lung adenocarcinoma. *Front Mol Biosci.* 2021;8:688274. doi: 10.3389/fmolb.2021.688274.
31. Huang W, Huang H, Zhang S, Wang X, Ouyang J, Lin Z, et al. A novel diagnosis method based on methylation analysis of SHOX2 and serum biomarker for early stage lung cancer. *Cancer Control.* 2020;27(1):1073274820969703. doi: 10.1177/1073274820969703.
32. Zeng S, Lin C, Huang Y. miR-375 Combined with SHOX2 Methylation has higher diagnostic efficacy for non-small-cell lung cancer. *Mol Biotechnol.* 2023;65(7):1187-97. doi: 10.1007/s12033-022-00604-y.
33. Vo TTL, Nguyen TN, Nguyen TT, Pham ATD, Vuong DL, Ta VT, et al. SHOX2 methylation in Vietnamese patients with lung cancer. *Mol Biol Rep.* 2022;49(5):3413-21. doi: 10.1007/s11033-022-07172-z.
34. Kneip C, Schmidt B, Seegebarth A, Weickmann S, Fleischhacker M, Liebenberg V, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J Thorac Oncol.* 2011;6(10):1632-8. doi: 10.1097/JTO.0b013e318220ef9a.
35. Feng H, Shao W, Du L, Qing X, Zhang Z, Liang C, et al. Detection of SHOX2 DNA methylation by methylation-specific PCR in non-small cell lung cancer. *Transl Cancer Res.* 2020;9(10):6070-7. doi: 10.21037/tcr-20-887.

Table 1. Baseline data and clinicopathological characteristics of patients with lung cancer

Number of samples	50	23 samples <50
		27 samples >50
Age (mean)	56.7	
Gender	Female	24
	Male	26
Stage	Stage 2	23
	Stage 3	25
	Stage 4	2
Lymph node metastasis	Negative	21
	Positive	29

Table 2. Primer sequencing

	Forward and reverse	Annealing temperature (°C)
FTH_1	Forward:GATCCTGAGTATGGATGAA Reverse:CGTTTGCCATGCCAGAG	60
SHOX	Forward:GTCCGTAATCGTACGAATC Reverse:AATCGTGATCGGAAATGCGA	60

Table 3. Clinicopathological characteristics of patients Coloration with FTH-1and SHOX2 genes

	FTH-1	SHOX2
Age	<i>P</i> value:0.56	<i>P</i> value:0.182
Sex	<i>P</i> value:0.231	<i>P</i> value:0.324
Stage	<i>P</i> value:0.043	<i>P</i> value:0.031
Lymph node metastasis	<i>P</i> value:0.029	<i>P</i> value:0.042

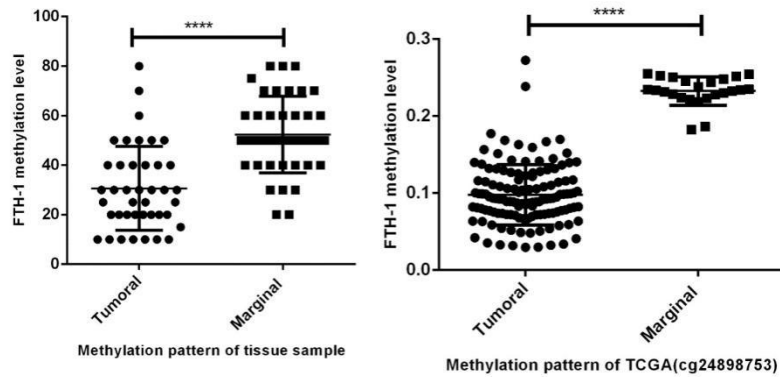


Figure 1. This figure shows the analysis of methylation levels of FTH-1 in NSCLC samples using q-MSP. The results showed that the gene is significantly hypomethylated in NSCLC tumors in comparison with normal adjacent tissues; $P < 0.05$. FTH-1 methylation status in TCGA-STAD. Comparison of FTH-1 overall methylation levels between NSCLC and normal samples using TCGA-STAD methylation cohort. FTH-1 shows significantly hypomethylated in NSCLC tumors ($**** P < 0.0001$).

NSCLC: Non-small cell lung cancer; FTH-1: Ferritin Heavy Chain 1; q-MSP: Quantitative Methylation-specific PCR; TCGA-STAD: The Cancer Genome Atlas- Stomach adenocarcinoma

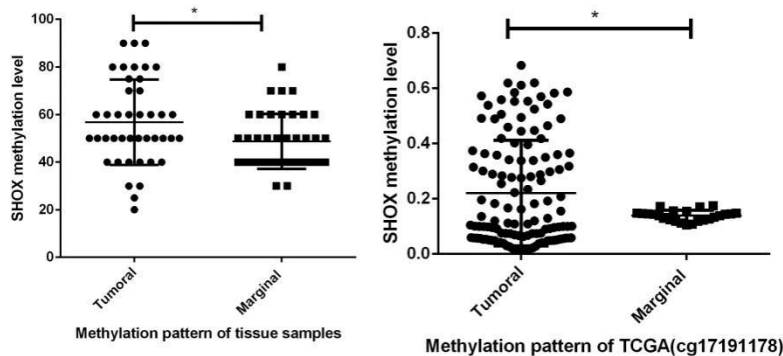


Figure 2. Analysis of SHOX2 methylation levels in NSCLC samples using q-MSP. The results show that the gene is hypermethylated in NSCLC tumors in comparison with normal adjacent tissues (*: $P < 0.05$). SHOX2 methylation status in TCGA-STAD. Comparison of SHOX2 hyper methylation levels between NSCLC and normal samples using TCGA-STAD methylation cohort. SHOX2 shows significantly hypermethylated in NSCLC tumors (*: $P < 0.05$).

NSCLC: Non-small cell lung cancer; SHOX2: Short stature homeobox 2; q-MSP: Quantitative methylation-specific PCR; TCGA-STAD: The Cancer Genome Atlas- Stomach adenocarcinoma

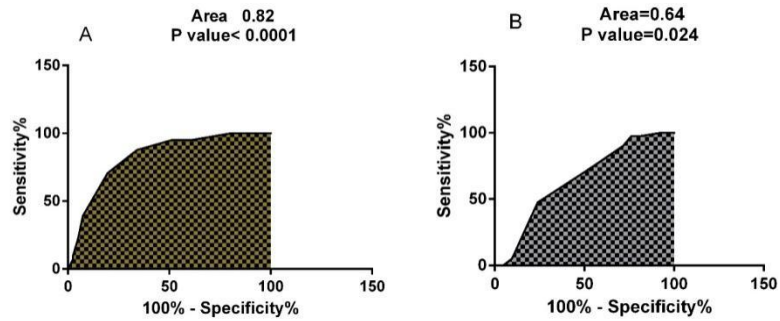


Figure 3. ROC curve analysis; A) ROC curve analysis was performed on NSCLC samples for FTH-1 that shows a promising diagnostic value. B) ROC curve analysis was performed on NSCLC samples for SHOX2 accuracy as a diagnostic biomarker for NSCLC as well. ROC: Receiver operating characteristic; NSCLC: Non-small cell lung cancer; SHOX2: Short stature homeobox 2. q-MSP: Quantitative methylation-specific PCR; FTH-1: Ferritin Heavy Chain 1