


GNPNAT1 Predicts Poor Prognosis and Cancer Development in Non-Small Cell Lung Cancer

Yong Feng, Na Li , Yi Ren

Department of Thoracic Surgery, Shenyang Chest Hospital, the Tenth People's Hospital of Shenyang, Shenyang, People's Republic of China

Correspondence: Yi Ren, Shenyang Chest Hospital, the Tenth People's Hospital of Shenyang, 11 Beihai Street, Shenyang, 110044, People's Republic of China, Tel +8615942068710, Email yi498857876@163.com

Background: Glucosamine-phosphate N-acetyltransferase 1 (GNPNAT1) is a key enzyme in the biosynthetic pathway of uridine diphosphate-N-acetylglucosamine and is upregulated in multiple malignancies. However, its function in cancer biology remains unclear.

Methods: Using TCGA dataset, this study analysed GNPNAT1 expression in non-small cell lung cancer (NSCLC) and assessed the correlation between GNPNAT1 and NSCLC patient prognosis. MTT and transwell assays were performed to determine the effect of GNPNAT1 on the growth and metastatic ability of lung cancer cells. GNPNAT1 expression was detected using immunohistochemistry in 78 NSCLC patients, and we analysed the correlation among clinicopathological parameters, overall survival (OS) and GNPNAT1 levels. Transcription factors that potentially regulate GNPNAT1 were explored using database analysis. RNF2 expression was verified using immunohistochemistry in NSCLC tissues.

Results: The results indicated that GNPNAT1 was upregulated in NSCLC, and patients with high GNPNAT1 levels had a poor prognosis. GNPNAT1 overexpression promoted the proliferative and metastatic ability of lung cancer cells, whereas GNPNAT1 knockdown showed the opposite effect. GNPNAT1 expression was upregulated in NSCLC tissues compared to matched normal tissues as assessed by immunohistochemistry. Moreover, GNPNAT1 levels were positively correlated with histological type and pathological stage. The negative correlation between GNPNAT1 levels and OS was confirmed in 78 NSCLC patients. Aberrant RNF2 partly contributed to the upregulation of GNPNAT1 expression in NSCLC.

Conclusion: These findings suggested that GNPNAT1 was upregulated and played an important role in NSCLC. GNPNAT1 is expected to represent an effective prognostic biomarker for NSCLC patients.

Keywords: non-small cell lung cancer, glucosamine-phosphate N-acetyltransferase 1, overall survival, proliferation, metastasis

Introduction

Lung cancer is a commonly diagnosed cancer worldwide with an estimated 1.8 million new lung cancer cases diagnosed each year based on global cancer statistics.¹ Approximately 85% of lung cancers are classified as non-small cell lung cancer (NSCLC), which is mainly composed of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC).² Over the past few decades, substantial progress has been made in the screening, diagnosis and treatment of lung cancer.³ However, this progress has been far from sufficient to reduce the mortality of NSCLC. Understanding the pathogenesis of lung cancer is necessary to identify more effective screening strategies and therapies.

Glucosamine-phosphate N-acetyltransferase 1 (GNPNAT1) is a key enzyme in the biosynthetic pathway of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which plays a key role in the N-linked glycosylation of proteins and the generation of glycosyl phosphatidylinositol linkers.⁴ Inactivation of murine GNPNAT1 by homologous recombination reduces the amount of UDP-GlcNAc, therefore resulting embryonic death and the proliferation defective cells.^{5,6} In *Saccharomyces cerevisiae*, GNPNAT1 is necessary for cell survival.⁷ GNPNAT1 can be detected in most tissues in humans. The correlation between GNPNAT1 and cancer was first reported in prostate cancer.⁸ In recent years, several studies have been published sequentially and indicated that GNPNAT1 upregulation predicts poor prognosis in LUAD patients.^{9–12} Zhao et al found that Abraxane was a promising drug to treat NSCLC, and the application of Abraxane

resulted in significant downregulation of GNPAT1 in lung cancer cells.¹³ GNPAT1 might play an important role in NSCLC biological processes and represent an underlying biomarker for prognosis.

In this study, we assessed the expression and prognostic value of GNPAT1 in NSCLC patients, detected the possible function of GNPAT1 in NSCLC development and analysed the underlying mechanism for GNPAT1 upregulation in NSCLC. These results potentially uncover a valuable target or biomarker for NSCLC.

Materials and Methods

Dataset and Processing

The NSCLC cases in this study included LUAD and LUSC. GNPAT1 mRNA expression data and prognostic information were obtained from TCGA and GEO databases. The datasets used in this study provided clinical stage information. These datasets were used to analyse the differential expression of GNPAT1 between NSCLC and normal tissues and the correlation between GNPAT1 levels and overall survival (OS). The transcription factors (TFs) regulating GNPAT1 were screened using The Signalling Pathways Project (<http://www.signallingpathways.org/omni/ner/query.jsf>). We utilized TCGA database to analyse the correlation between GNPAT1 and TFs in four datasets (LUSC tumours, LUSC tumour and lung tissues, LUAD tumours, LUAD tumour and lung tissues). Then, we obtained the intersection of the ten TFs with the highest correlation with GNPAT1 in four datasets and finally identified four TFs that appeared in four datasets simultaneously. RNF2 mRNA levels in LUAD and LUSC were analysed using GEPIA (<http://gepia.cancer-pku.cn/>).

Patients

This study included 35 LUAD patients and 43 LUSC patients who received surgical treatment between May 2016 and August 2020 at Shenyang Chest Hospital. The cancer tissues and matched normal lung tissues were obtained from surgical excision. The mean age of the patients was 61.91 ± 9.33 years. All tissues were conventionally fixed with formaldehyde and embedded in paraffin. The diagnoses for all samples were confirmed by two pathologists. No patient received other therapy before surgery. All patients submitted their informed consent before enrolment. This study was approved by the Ethics Committee of Shenyang Chest Hospital. Our study complied with the Declaration of Helsinki.

Cell Construction

The ORF of GNPAT1 was cloned into the pLVX-Puro vector (primers for GNPAT1 overexpression: F-gaattcGCCTCTACGGACCTTACT, R-tctagaATTTATGGAGGTCACCTCG). The shRNA sequence targeting GNPAT1 was inserted into PLKO (primers for GNPAT1 knockdown: F-CCGGGAGTCAGAATACAGCTACA TTCTCGAGAATGTAGCTGTATTCTGACTCTTTTG, R-AATTCAAAAAGAGTCAGAATACAGCTACATTCTCG AGAATGTAGCTGTATTCTGACTC). Scramble sequences were inserted into vectors to function as controls, including PLVX or negative controls (NCs). These vectors were co-transfected into 293TN cells with packing vectors to obtain pseudolentiviral particles, which were used to infect A549 (lung adenocarcinoma cells) and NCI-H266 (lung squamous carcinoma cells) cells. Cells were purified with 2 µg/mL puromycin (Beyotime, Shanghai, China). All the cells were cultured in RPMI-1640 (GIBCO, USA) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 µg/mL streptomycin (Beyotime), and 100 IU/mL penicillin (Beyotime) at 37 °C in a humidified atmosphere containing 5% CO₂.

Real-Time PCR

Total RNA was extracted from lung cancer cells using TRIzol[®] (Invitrogen) and then reverse transcribed into cDNA using a reverse transcription kit (Trans Gen Biotech Co, Beijing, China). GNPAT1 was quantified using the SYBR Green PCR Mix (Bio Researcher, Beijing, China). The reaction included 38 cycles, and the cycle included denaturation for 10s at 95 °C and annealing and prolongation for 30s at 60 °C. GAPDH served as the endogenous control. Each sample was tested in triplicate. All assays were replicated thrice. The following primers with sequences were employed:

GNPNAT1, F-ACCCAAGTCTACTCAAAG and R-TCAGCAGTACAAAGAGGC; GAPDH, F-ATGGGGAAGGTG AAGGTCG and R-GGGGTCATTGATGGCAACAATA.

MTT Assay

Cells were seeded into 96-well plates and cultured for 4, 24, 48 and 72 h. Then, 20 μ L of MTT solution (Beyotime) was added to the well, and the samples were incubated for 4 h. The absorbance at 492 nm was measured using a microplate reader. The relative proliferative capacity was reflected based on the OD value. The OD value obtained at 4 h after inoculation represented the number of cells at inoculation.

Transwell Assay

A total of 3×10^4 cells in 500 μ L serum-free medium were seeded into the upper chamber coated with or without Matrigel (Corning Life Sciences, Tewksbury, MA, USA). Then, 700 μ L medium containing 10% FBS was added to the lower chamber. After culturing for 24 h, the chamber was placed into a 0.5% crystal violet solution (dissolved in methanol) to fix and stain the cells. The cells on the top side of the upper chamber were removed with a cotton swab, whereas the cells on the opposite side of the insert were counted using an inverted light microscope. Three high-power fields were selected to calculate the average number of cells to reflect the cell migration and invasion abilities.

Immunohistochemistry (IHC)

Briefly, 5- μ m-thick sections were cut from formalin-fixed and paraffin-embedded specimens. After deparaffinization and rehydration, the paraffin sections were treated with 3% H₂O₂ to block endogenous peroxidase at RT for 15 mins. Antigen retrieval was performed through microwave treatment (citrate buffer, pH 6.0). The nonspecific reaction was blocked with 5% bovine serum albumin (ZSGB Bio, Beijing, China) at RT for 15 mins. The sections were then incubated with rabbit polyclonal primary antibody against GNPAT1 (Proteintech, Wuhan, China, catalogue number: 16282-1-AP, 1:200 dilution) and rabbit polyclonal primary antibody against RNF2 (Affinity Biosciences, Liyang, China, catalogue number: DF7403, 1:100 dilution) at 4 °C overnight. A streptavidin-biotin peroxidase kit (ZSGB Bio) and DAB (ZSGB Bio) were used to visualise the antigen-antibody reaction.

The final immunohistochemical score for each sample was calculated using the following formula: intensity score \times the extent score. Staining intensity was quantified as follows: negative (0), weak (1), moderate (2), or strong (3). The staining extent was scored based on the quantity of positive cells: none (0), < 25% (1), 25–50% (2), 50–75% (3), or > 75% (4).¹⁴ Two pathologists evaluated the results in a blinded manner. The diverging scores were reviewed, and a consensus was reached between the two pathologists.

Statistical Analysis

The difference between two groups was analysed by a two-sided Student's *t* test. One-way ANOVA was used to analyse the correlation between GNPAT1 and clinical pathological parameters by screening logical independent factors. Then, multivariate linear stepwise regression was used to assess the association between GNPAT1 level and meaningful independent factors. The effect of GNPAT1 on overall survival (OS) was analysed using the Kaplan–Meier method through the Log rank test. The median relative mRNA level or IHC score was used as the cut-off value for high and low GNPAT1 mRNA group classification in different datasets. The correlation between GNPAT1 and TFs was analysed using the Pearson correlation coefficient. All the data are presented as the mean or median. $P < 0.05$ indicated statistical significance.

Results

GNPNAT1 mRNA Was Upregulated in NSCLC and Indicated a Poor Prognosis

We downloaded the GNPAT1 expression datasets of NSCLC, LUAD and LUSC from the GEO and TCGA databases to compare the differential expression of GNPAT1 mRNA between tumour and matched normal tissues. GNPAT1 expression was significantly upregulated in NSCLC as well as in LUSC and LUAD compared to normal tissues (Figure 1A and C). GNPAT1 mRNA levels increased with pathological stage from I to III/IV in NSCLC, LUSC and

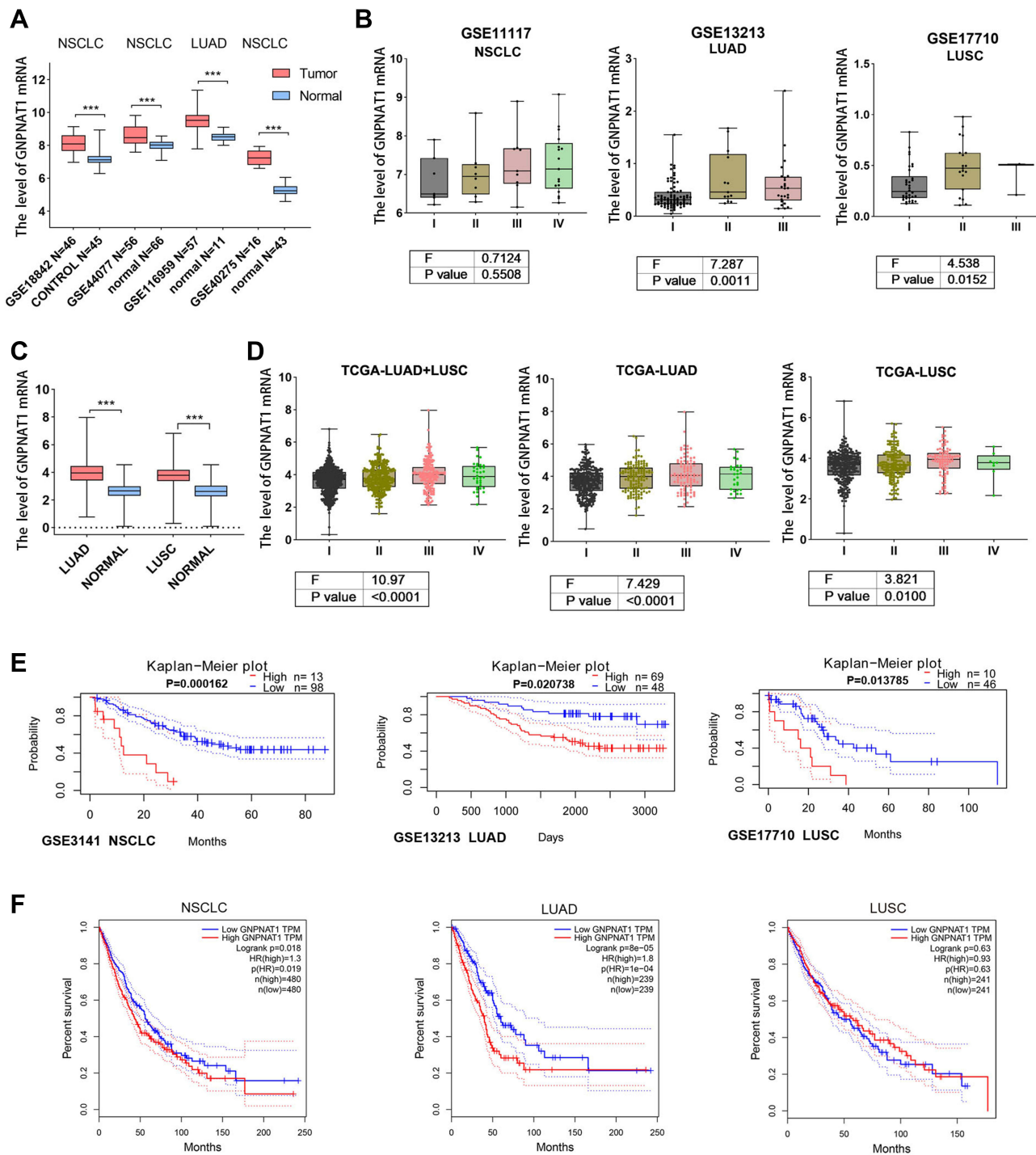


Figure 1 Dataset analysis. **(A and C)** The mRNA level of GNPAT1 was compared between cancer and normal tissues using the GEO dataset **(A)** and TCGA dataset **(C)**. **B and D.** GNPAT1 mRNA levels were analysed in cancer patients with different pathological stages in the GEO dataset **(B)** and TCGA dataset **(D)**. **(E and F)** Patients were divided into high and low groups according to the expression of GNPAT1 in lung cancer, and then OS was compared between the two groups using the GEO dataset **(E)** and TCGA dataset **(F)**. *** P<0.001.

LUAD **(Figure 1B and D)**. We used three GEO datasets to analyse the correlation between the GNPAT1 level and the prognosis of patients with lung cancer. The results indicated that NSCLC, LUSC and LUAD patients with higher levels of GNPAT1 had a shorter OS than those with lower levels of GNPAT1 **(Figure 1E)**. According to TCGA data analysis results, NSCLC patients with higher GNPAT1 levels had a poor prognosis **(Figure 1F)**. However, GNPAT1 was

a useful prognostic marker for LUAD patients but not for LUSC patients when the NSCLC patients were classified into LUAD and LUSC groups (Figure 1F). These results indicated that GNP NAT1 was upregulated in NSCLC tissues and might represent a biomarker to evaluate the prognosis of NSCLC patients, especially LUAD patients.

GNPNAT1 Improved the Proliferative and Metastatic Ability of Lung Cancer Cells

GNPNAT1 mRNA was detected by real-time PCR to verify the upregulation or knockdown of GNP NAT1 in A549 cells and NCI-H226 cells (Figure 2A). GNP NAT1 overexpression promoted cell proliferation, whereas knockdown of GNP NAT1 inhibited cell proliferation (Figure 2B). The abilities of GNP NAT1-overexpressing cells to migrate and invade were significantly improved, whereas these abilities were significantly decreased after GNP NAT1 knockdown in A549 (Figure 2C and D) and NCI-226 (Figure 2E and F) cells. The results showed that GNP NAT1 promotes the evolution of lung cancer cells.

GNPNAT1 Expression Was Increased in NSCLC Tissues and Correlated with Clinical Pathological Parameters

We detected GNP NAT1 expression in 78 NSCLC tissues (composed of 35 LUAD and 43 LUSC) and matched normal tissues by immunohistochemistry (Supplementary Figure 1). As shown in Figure 3A and B, GNP NAT1 was significantly upregulated in both LUAD and LUSC tissues compared to normal tissues. GNP NAT1 was localized in the cytoplasm (Figure 3A). GNP NAT1 was also expressed in normal tissues, but very weak expression was observed. Moreover, GNP NAT1 expression was increased in LUAD compared with LUSC (Figure 3B).

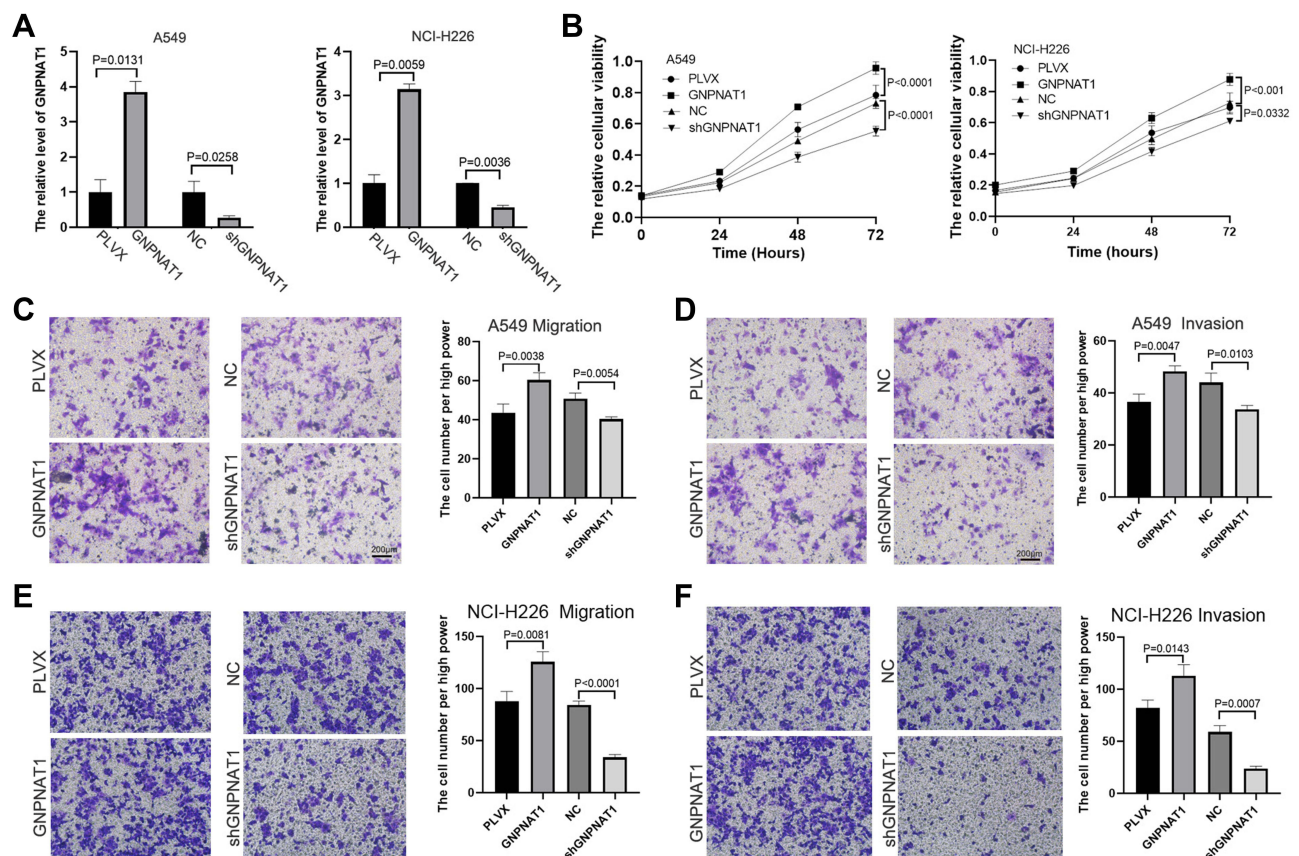


Figure 2 GNP NAT1 contributed to the evolution of lung cancer cells. (A) The mRNA level of GNP NAT1 was detected by real-time PCR. (B) The effect of GNP NAT1 on the proliferation of lung cancer cells was detected by MTT assay. (C and D) Transwell assays were used to detect the effect of GNP NAT1 on the migration (C) and invasion (D) of A549. (E and F) Transwell assays were used to detect the effect of GNP NAT1 on the migration (E) and invasion (F) of NCI-H226.

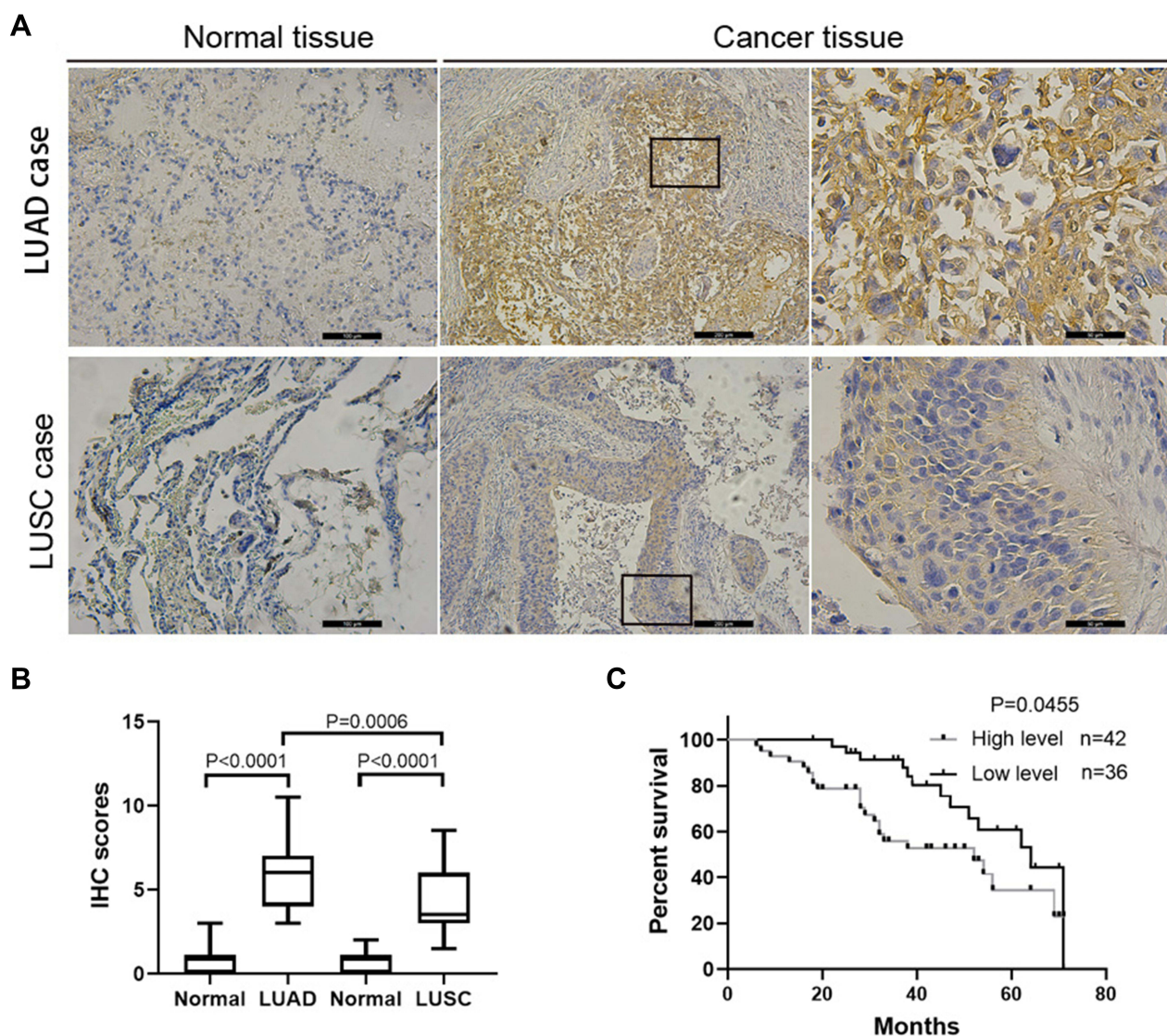


Figure 3 Immunohistochemistry assay. **(A)** GNPAT1 was upregulated in cancer tissues compared to normal tissues and was more highly expressed in LUAD than in LUSC. The image on the right is the enlarged image in the box. **(B)** The expression of GNPAT1 was quantified as a bar chart. **(C)** The patients with low levels of GNPAT1 (IHC score < 5) had a better OS than those with high levels of GNPAT1 (IHC score ≥ 5).

We analysed the correlation between GNPAT1 and clinical pathological parameters in 78 NSCLC patients. The results indicated that age, histological classification, and TNM stage were correlated with GNPAT1 expression (Table 1). Multivariate regression analysis confirmed that histological classification and TNM stage were two independent factors for GNPAT1 expression, whereas age was excluded (Table 2). The medium GNPAT1 staining score was higher in LUAD patients and TNM III–IV patients. Additionally, we analysed the correlation between GNPAT1 level and prognosis. The results indicated that the patients with low GNPAT1 levels (IHC score < 5) had a better OS than the patients with high GNPAT1 levels (IHC score ≥ 5), although the P value was marginal (Figure 3C).

These results suggested that GNPAT1 had a role in normal tissues. However, its expression was significantly increased in cancer tissues. GNPAT1 might have an important role during the development of NSCLC.

Table 1 Relationship Between GNP NAT1 Expression and Clinicopathological Parameters in NSCLC

Parameter	No. of Cases (%)	Median IHC Score	P value
Age (years)			0.04482
≤ 60	33	6	
> 60	45	4	
Gender			0.4453
Male	46	5	
Female	32	5	
Histological types			0.03454
LUAD	35	6	
LUSC	43	3.5	
Tumour differentiation			0.3819
High and moderate	56	5	
Low	22	5	
Lymph node metastasis			0.08384
Yes	22	6	
No	56	4.25	
Distant metastasis			0.2941
Yes	6	7	
No	72	4.75	
TNM stage			< 0.0001
I-II	56	3.75	
III-IV	22	7	

Table 2 Multivariate Analysis of Factors Related to GNP NAT1 Expression

	Estimate	Std. Error	z value	Pr (> z)
Intercept	-2.8164	0.7368	-3.822	0.000132
Histological types	2.3431	0.8018	2.922	0.003474
TNM stage	3.5192	0.8575	4.104	4.06E-05

GNPNAT1 Upregulation Was Associated with Transcriptional Regulation

To explore the mechanism of GNP NAT1 upregulation, we first predicted the underlying TFs targeting GNP NAT1. A total of 504 TFs were found to have the potential to regulate GNP NAT1 transcription. We analysed the correlation between GNP NAT1 with TFs in different datasets, including LUSC tumours, LUSC tumour and lung tissues, LUAD tumours, LUAD tumour and lung tissues. We took the intersection of the ten TFs with the highest correlation with GNP NAT1 in four datasets and finally obtained four TFs (Figure 4A). Moreover, RNF2, RAD21, YY1 and ZNF143 were positively correlated with GNP NAT1 in NSCLC consisting of LUAD and LUSC tissues (Figure 4B). Of them, RNF2 mRNA was upregulated in LUAD and LUSC (Figure 4C). We detected RNF2 expression in lung cancer tissues (Supplementary Figure 1) and found that RNF2 located in both cytoplasm and nucleus (Figure 4D). Considering that RNF2 mainly acts as a transcription factor, we focused on the expression of nucleic RNF2. The result indicated that nucleic RNF2 was not statistically different between LUAD and LUSC (Figure 4E). Moreover, RNF2 was positively correlated with GNP NAT1 in 78 cases of NSCLC (Figure 4F). In most cases, GNP NAT1 expression was higher in tissues with higher RNF2 expression, and lower in tissues with lower RNF2 expression. Thus, the upregulation of some TFs in NSCLC might represent one of the mechanisms for elevated GNP NAT1 expression.

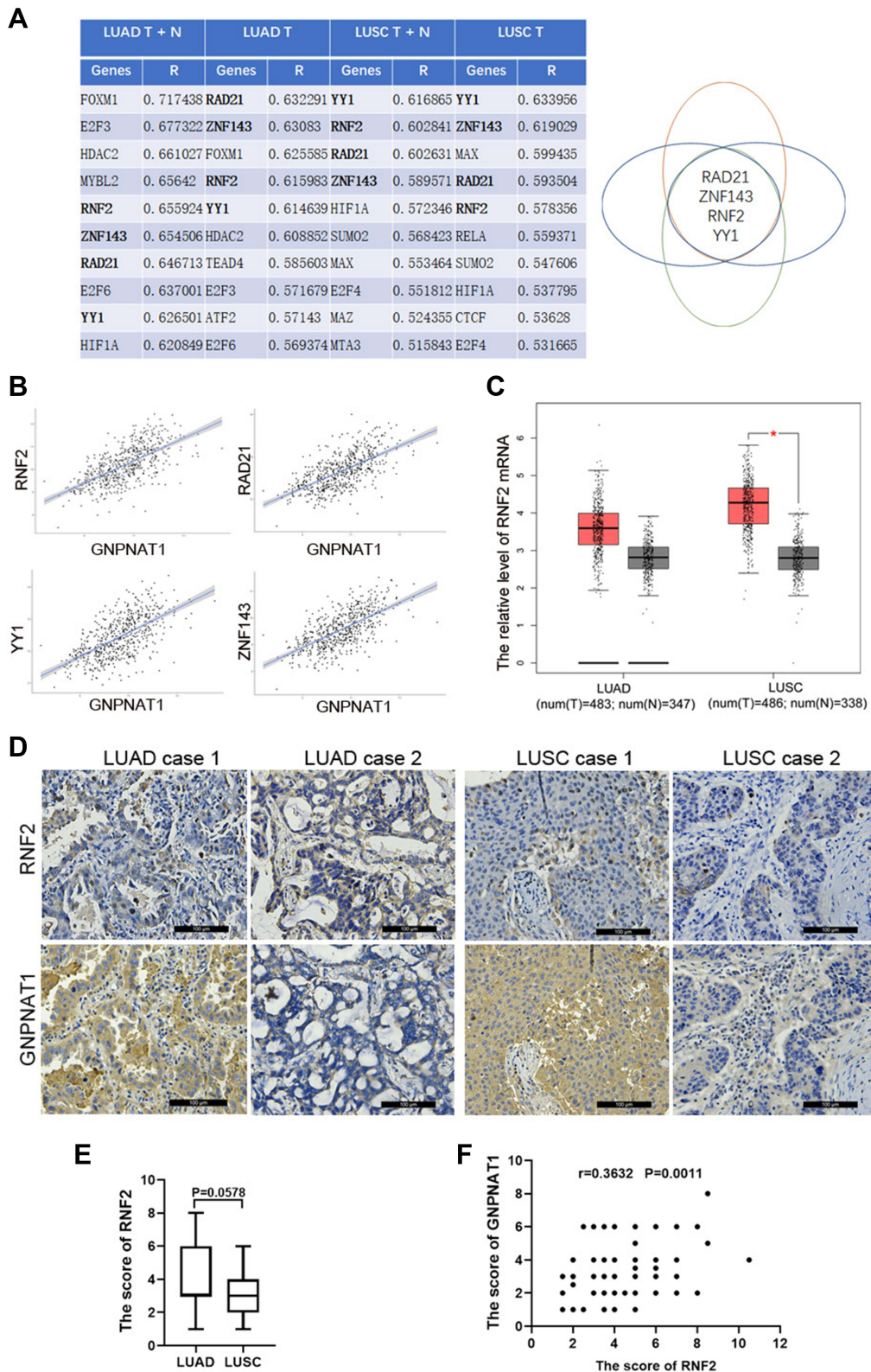


Figure 4 Aberrant TFs contributed to the upregulation of GNPAT1 in NSCLC. **(A)** RNF2, ZNF143, RAD21, and YY1 were the intersecting TFs from the top ten TFs most correlated with GNPAT1 in the four groups. **(B)** The four TFs were highly positively correlated with GNPAT1 in LUAD and LUSC tissues. **(C)** RNF2 mRNA was increased in LUAD and LUSC tissues. **(D)** IHC assays indicated that RNF2 located in both cytoplasm and nucleus of lung cancer cells. **(E)** The nucleic RNF2 was not statistically different between LUAD and LUSC. **(F)** The nucleic RNF2 was positively correlated with GNPAT1 in NSCLC. * $P < 0.05$.

Discussion

The glucosamine-6-phosphate acetyltransferase GNPAT1 catalyses the reaction of glucosamine 6-phosphate with acetyl-CoA to form the precursor of UDP-GlcNAc, which plays a key role in the N-linked glycosylation of proteins.^{5,15} As previously reported, N-linked glycosylation is a posttranslational modification occurring in many eukaryotic secreted and surface-bound proteins that impacts the function of these proteins.¹⁶ UDP-GlcNAc is also involved in the generation of glycosyl phosphatidylinositol linkers, through which membrane proteins anchor to the cell.¹⁷ Thus, GNPAT1 might function in multiple physiological and pathological processes by affecting UDP-GlcNAc formation.

The critical function of GNPAT1 has been verified in different species. Toshiyuki et al found that a GNPAT1-deletion mutant resulted in the reduced virulence of *Candida albicans*.⁷ The murine GlcNAc EMeg32 was first cloned in 2000.⁵ Inactivation of EMeg32 downregulated UDP-GlcNAc levels and subsequently led to embryonic death and reduced proliferation in embryonic stem cells and embryonic fibroblast cells.⁶ Recently, a study found that GNPAT1 was necessary for growth plate chondrogenesis in humans.¹⁸ Knockdown of GNPAT1 decreased cellular proliferation and the expression of chondrocyte differentiation markers.¹⁸

To date, a few studies have explored the function of GNPAT1 in cancer. According to the results of Akash et al, GNPAT1 was significantly elevated at the transcript and translational levels in prostate cancer compared with benign tissues, whereas lower levels were noted sites of lymph node metastasis and in castration-resistant prostate cancer (CRPC) tissues compared with localized prostate cancer.⁸ Loss of GNPAT1 in CRPC-like cells resulted in increased proliferation and aggressiveness by activating the PI3K-AKT pathway.⁸ GNPAT1 along with other metabolism-related genes correlate with the poor prognosis of LUAD patients, and an independent prognostic model was established based on these genes.^{19,20} GNPAT1 overexpression potentially correlated with DNA copy amplification, low DNA methylation and loss of miRNA.¹⁰ Here, we showed that the upregulation of some TFs was also responsible for increased GNPAT1 expression. The results of GNPAT1 gene coexpression network analysis indicated that GNPAT1 might participate in cell cycle transition, cancer development and immune infiltration in LUAD.¹¹ The other two studies also reported a correlation between GNPAT1 and immune infiltration, indicating the potential function of GNPAT1 in immunomodulation and immunotherapy.^{9,21}

In this study, we analysed GNPAT1 expression in NSCLC. The results showed that GNPAT1 was upregulated in NSCLC and associated with the poor prognosis of NSCLC patients. Similar results were obtained when we divided NSCLC patients into LUAD and LUSC subcategories. Several recent studies also reached consistent conclusions about GNPAT1 in LUAD.⁹⁻¹² However, few studies have reported the function of GNPAT1 in NSCLC or LUSC. In addition, GNPAT1 represents the key molecule facilitating the enhanced therapeutic effects of Abraxane on NSCLC.¹³ Abraxane downregulated the expression of GNPAT1 and led to impaired proliferation in NSCLC cell lines.¹³ In this study, we identified the expression of GNPAT1 protein in NSCLC tissues, detected its function in lung cancer cells, and consequently provided experimental evidence for GNPAT1 function. Moreover, we found that GNPAT1 was more valuable as a prognostic marker in LUAD patients. It is possible that GNPAT1 expression was higher in LUAD patients than in LUSC patients according to the immunohistochemistry results. Our study revealed the potential function of GNPAT1 in NSCLC. However, the mechanism of GNPAT1 has not been clearly defined, which limits the clinical application of GNPAT1. The value of GNPAT1 as a prognostic biomarker needs to be confirmed in more patients. In the future, we will explore the mechanism by which GNPAT1 exerts its effect on NSCLC and its potential in clinical prognosis assessment and treatment.

Conclusion

This study found that GNPAT1 was significantly upregulated in NSCLC compared with normal lung tissues, and the effect was partly attributed to the aberrant TFs. Elevated GNPAT1 levels are associated with the tumour histological type, stage, and poor prognosis of patients. GNPAT1 might play an important role in NSCLC development and represent an underlying therapeutic target. More studies are needed to uncover the function of GNPAT1 in cancer.

Abbreviations

GNPAT1, glucosamine-phosphate N-acetyltransferase 1; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; UDP-GlcNAc, uridine diphosphate-N-acetylglucosamine; OS, overall survival.

Ethical Statement

All patients submitted their informed consent before enrolment. This study was approved by the Ethics Committee of Shenyang Chest Hospital.

Acknowledgments

Not available.

Disclosure

The authors report no conflicts of interest in this work.

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